Trop J Nat Prod Res, December 2023; 7(12):5501-5508 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Investigation of the Inflammatory, Antipyretic, and Analgesic Potential of Ethanol Extract from *Hedyotis capitellata* Wall. ex G. Don Leaves in Mice

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ARTICLE INFO

Article history:
Received 09 September 2023
Revised 10 October 2023
Accepted 19 October 2023
Published online 01 January 2024

ABSTRACT

Inflammation, fever, and pain are common symptoms that can adversely affect overall health and quality of life. *Hedyotis capitellata* Wall. ex G. Don (Rubiaceae) is a traditional herbal medicine that alleviates these symptoms. However, scientific evidence supporting its effectiveness remains limited. Therefore, this study aimed to evaluate the potential of *H. capitellata* in controlling pain, fever, and inflammation. The analgesic properties of *H. capitellata*, were assessed using acetic acid and formalin tests. Paw edema induced by 1% carrageenan was used to evaluate anti-inflammatory activity, while the yeast-induced fever model was employed to investigate the antipyretic activity of *H. capitellata*. Ethanol leaf extract of *H. capitellata* (EHCW) was administered at different doses of 200, 250, and 300 mg/kg, with positive control groups receiving aspirin, tramadol, indomethacin, and paracetamol, and a negative control group receiving normal saline. The results showed that ethanol leaf extract of *H. capitellata* exhibited the highest analgesic activity ($p < 0.05$) at 300 mg/kg. It also demonstrated significant inhibition of paw edema ($p < 0.05$), at 300 mg/kg. Ethanol leaf extract of *H. capitellata* demonstrated a significant reduction ($p < 0.05$) in rectal temperature in the yeast-induced fever model, with the maximum effect at 300 mg/kg. In conclusion, the study demonstrated the potential of *H. capitellata* in treating conditions related to inflammation, pain, and fever.

Keywords: Analgesic, anti-inflammatory, antipyretic, *Hedyotis capitellata* Wall. ex G. Don

Introduction

Pain is a complex emotional and sensory state often associated with tissue damage. Despite its unpleasant nature, its function within the body is protective, serving to provide warning signals of injury or potential harm to the organism. Pain involves intricate interactions of multiple factors, including sensory receptors, nerve conduction, nerve fibers, neural pathways, and specific anatomical locations within the body. Physiologically, pain begins when sensory nerve cells are stimulated beyond their threshold (pain-sensing organs capable of detecting harmful signals). It is modulated through specific transmission mechanisms at their peripheral terminals, transmitting signals to the central nervous system (CNS) via specialized neural networks. Pain can be inflammatory or non-inflammatory, depending on the response to tissue injury. Medications for pain control include opioids, nonsteroidal anti-inflammatory drugs, etc.[1]

Inflammation is an initial defensive response of the immune system to infection or cellular and tissue damage. This activity is geared towards countering stimulating agents, invasive infections, immune system dysregulations, or neural degeneration. The alterations occurring in the state of acute inflammation play a pivotal role in maintaining the organism's survival. Various types of cells participate in the inflammatory process, including macrophages, neutrophils, lymphocytes, and mast cells.

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Citation: Nhung TTP and Quoc LPT. Investigation of the Inflammatory, Antipyretic, and Analgesic Potential of Ethanol Extract from *Hedyotis capitellata* Wall. ex G. Don Leaves in Mice. Trop J Nat Prod Res. 2023; 7(11):5501-5508. http://www.doi.org/10.26538/tjnpr/v7i12.20

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When these cells are attracted to the site of injury, they generate various inflammatory mediators such as cytokines, histamine, nitric oxide (NO), prostaglandins, leukotrienes, and other substances. Changes in the endothelial cell junctions in the bloodstream allow serum proteins to infiltrate tissues, leading to characteristic inflammatory signs like increased blood flow, redness, warmth, fluid leakage into tissues, and swelling. Non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, glucocorticoids, and various other agents have been widely employed to effectively reduce inflammation.[2]

Fever is a condition characterized by an elevation in body temperature, typically stemming from inflammation, infection, malignancies, or tissue damage. When tissues are affected by disease or injury, they initiate the production of inflammatory mediators such as cytokines (including interleukin 1β, α, β, and TNF-α). This, in turn, leads to an increased synthesis of prostaglandin E2 (PGE2) in the vicinity of blood vessels in the lower hypothalamic region. This stimulation triggers the thermoregulatory center in the lower hypothalamus, resulting in a rise in body temperature. Fever-reducing medications like acetaminophen, efferalgan, and similar agents often act by inhibiting or suppressing the expression of the COX-2 enzyme to reduce elevated body temperature through the inhibition of PGE2 production.[3]

The use of pain relievers, anti-inflammatory drugs, and antipyretics provides certain effectiveness. However, their frequent and long-term use can lead to various side effects such as gastrointestinal irritation, cardiovascular toxicity, tolerance, and dependence. Furthermore, most patients do not achieve complete pain relief even when using multiple types of pain relievers and anti-inflammatory drugs.[3] Medicinal plants have the potential to provide novel compounds that interact with biological systems. Therefore, researching traditional herbal medicines used for anti-inflammatory, antipyretic, and analgesic purposes is considered a strategy for discovering new effective and rational drugs. Despite the availability of many synthetic anti-inflammatories,

antipyretic, and analgesic drugs in the market, the search for new drugs with good safety profiles remains significant.[4]

Hedyotis capitellata Wall. ex G. Don is a plant species commonly found in Vietnam's central and mountainous provinces. This plant is easy to cultivate, extract, process, and utilize. In traditional medicine, *H. capitellata* is well-known for its anti-inflammatory, diuretic, hemostatic, analgesic, anti-arthritic, and anti-gastritis properties. Moreover, *H. capitellata* has been shown to possess antioxidant, anti-inflammatory, and antibacterial effects. The leaf extract of *H. capitellata* contains alkaloids, saponins, tannins, and anthraquinones.[5] While the leaves of *H. capitellata* are frequently employed in traditional medicine for group of various illnesses, as far as our information goes, there is a lack of reports regarding the analgesic, antipyretic, and anti-inflammatory properties of this plant on animals. As a result, it has become essential to undertake scientific research into the analgesic, antipyretic, and anti-inflammatory characteristics of this plant. Thus, the primary objective of this study is to explore the analgesic, antipyretic, and anti-inflammatory effects of *H. capitellata*'s ethanol leaf extract using a mouse model.

Material and Methods

Collection plant material and preparation of the extract

Collection and preparation of plant material

The leaves of *H. capitellata* were collected by the authors from Cam My district, Dong Nai province, Vietnam (Coordinates: 10°48′27″N 107°14′21″E), in November 2022. Specimens were stored at the Plant Biotechnology Laboratory, Institute of Biotechnology and Food Technology, Ho Chi Minh City University of Industry, Vietnam, with voucher number HC221122VST. Fresh leaves were washed with clean water, air-dried in the shade, and further dried in a Memmert drying cabinet until the moisture content reached < 12%. The dried leaves were then ground into a fine powder and separated with a sieve (hole diameter: 0.18 mm).

Preparation of the extract

The leaf powder (250 g) was macerated in 800 mL of 96% ethanol for 72 h, with the solution being periodically shaken using a mini orbital shaker at a speed of 120 rpm during the maceration process. The resulting mixture was then roughly filtered through a clean, sterilized, white cotton pad and a Whatman No. 4 filter paper. The obtained extract (Ethanol leaf extract of *H. capitellata*) was concentrated using a rotary evaporator at 40 rpm and 55°C for 1 h. The extract solution was transferred to a glass vial, appropriately labeled, and stored in a desiccator over silica gel until use.

Phytochemical screening and determination of total phenolic (TPC), total flavonoid (TFC) of extract

Phytochemical screening of extract

Phytochemical analysis was carried out following standard protocols to qualitatively ascertain the presence of a range of compounds (tannins, saponins, flavonoids, alkaloids, phenols, glycosides, steroids, and terpenoids) in the ethanol leaf extract of *H. capitellata*.[6-9]

Determination of total phenolic (TPC), total flavonoid (TFC)

To determine the total phenolic content, we followed the Folin-Ciocalteu colorimetric method initially outlined by Iqbal *et al*. but with some adjustments.[10] Specifically, we combined 0.3 mL of the extract with 2.25 mL of Folin-Ciocalteu phenol reagent. Following a 5-minute incubation period, we introduced 2.25 mL of 6% sodium carbonate into the mixture, allowing it to sit at room temperature for 90 min. Subsequently, we measured the absorbance of the solution at 725 nm. A standard curve for gallic acid was also established within the concentration range of 0-200 µg/mL using a similar procedure. The findings were expressed as milligrams of gallic acid equivalents (GAE) per gram of the extract.

The total flavonoid content was assessed utilizing the aluminum colorimetric method, which was originally established by Iqbal *et al*. but with certain adjustments.[10] Quercetin served as the reference standard, and a calibration curve for quercetin was generated within the concentration range of 0-200 µg/mL. For this analysis, 0.5 mL of the extract and 0.5 mL of the standard were each placed into separate test tubes. To each tube, 10% aluminum chloride (0.1 mL), 1 M potassium acetate (0.1 mL), 80% methanol (1.5 mL), and distilled water (2.8 mL) were added and thoroughly mixed. A blank sample was similarly prepared, substituting 0.5 mL of distilled water for the sample or standard, and replacing the aluminum chloride with distilled water. All tubes were allowed to incubate at room temperature for 30 min, after which the absorbance was measured at 415 nm. The flavonoid concentration was expressed as milligrams of quercetin equivalents (QE) per gram of the extract.

Experimental animals

Healthy Swiss mice, aged 6-7 weeks and weighing 25-30 g, were procured from the Pasteur Institute in Ho Chi Minh City, Vietnam, for use in the experimental protocols. To mitigate stress, a 7-day acclimatization before the laboratory environment was provided for all animals prior to the commencement of any experimental procedures. The mice were individually housed in glass cages and maintained under standardized environmental conditions within a room set at of 24°C ± 2°C, with humidity levels between 50% and 60%, and a 12-hour light/12-hour dark cycle. Throughout the study, the mice were granted unrestricted access to a standard pelletized diet formulated for rodents, as well as ad libitum access to water. Animal group and handling adhered to the guidelines outlined by the World Health Organization (2000) [11] and followed the Helsinki Declaration, as per the description provided by Hurst.[12] All animal-related procedures and care were executed by trained personnel, in strict compliance with the regulations governing the use and welfare of laboratory animals in Vietnam, as well as the Ministry of Health of Vietnam's Guidelines on Ethics in Biomedical Research.[13] The study was approved by the Ethics Committee of the Council for Scientific Research and Technology Development of the Ho Chi Minh City University of Industry, Vietnam.

Assessment of analgesic activity

Experiment design

For each experimental model, the mice were distributed randomly into five groups, each consisting of five mice. The initial group functioned as the negative control (saline group) and was administered a solution of normal saline (10 mL/kg). The second group, denoted as the positive control (aspirin/tramadol group), was subjected to standard medications, which included the oral or injectable administration of aspirin (150 mg/kg) and tramadol (10 mg/kg) in experiments related to pain reduction induced by formalin and the writhing test triggered by acetic acid. The experimental groups with ethanol leaf extract of *H. capitellata* ($EHCW_{200}$, $EHCW_{250}$, and $EHCW_{300}$ groups) were administered EHCW at corresponding doses of 200 mg/kg, 250 mg/kg, and 300 mg/kg.

The writhing test induced by acetic acid

The writhing test, aimed at assessing the peripheral analgesic effects of the extract, was carried out following Chang *et al*.'s procedure with slight adjustments.[14] Writhing responses were induced by intraperitoneal injection of acetic acid (0.6% v/v) at a dose of 10 mL/kg, administered 30 min after the administration of the extract, vehicle (normal saline, 10 mL/kg), or the standard drug (aspirin, 150 mg/kg) to the respective groups of mice. The analgesic activity of the extract was gauged by tallying instances of writhing, characterized by "abdominal contractions and elongations" as well as "extension of hind limbs," within 30-minute, following a 5-minute delay. The reduction in the number of writhing episodes in comparison to the control group was indicative of the extract's analgesic potential. It is quantified as the percentage inhibition of writhing (PIW), calculated as follows:

$$\text{PIW (\%)} = \frac{Mean\ writhing\ count\ (control - test)}{Mean\ writhing\ count\ control} \times 100$$

Formalin-induced pain test

The formalin test was conducted following a modified protocol based on Anafi *et al*.[15] Pain was induced by injecting 20 µL of 5% formalin in distilled water into the subplantar region of the right hind paw. The duration (in seconds) of licking and biting the injected paw served as

an indicator of pain response. The ethanol leaf extract of *H. capitellata* (at doses of 200, 250, and 300 mg/kg) and tramadol (10 mg/kg) were administered 30 min before formalin injection. The control group received an equivalent volume of normal saline. Pain responses were measured during both the early phase (0-5 min after formalin injection) and the late phase (20-30 min after formalin injection). The duration of licking and biting the paw was observed, recorded, and expressed as a percentage of inhibition (PIF).

$$\text{PIF (\%)} = \frac{Latency\ (test) - Latency\ (control)}{Latency\ (test)} \times 100$$

The levels of cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE2) expression during the formalin test

Blood samples were collected from mice in the formalin test at both 0 and 30 min using the retro-orbital puncture technique. Serum was obtained by centrifugation at approximately 12,000 rpm for 5 min. ELISA kits for COX-2 and PGE2, supplied by Absolute Biotech Co., Ltd., were employed for individual measurements. Samples and standards were introduced into ELISA wells pre-coated with COX-2 and PGE2 antibodies, and HRP conjugate was subsequently added at 37°C. After 1 hour of incubation, each well underwent three washes with a washing solution. Each experiment was carried out independently three times. Reagent A and reagent B were added to the solutions and kept in an incubator at 37°C for 15-30 min. Finally, a stop solution was introduced to terminate the reaction. The absorbance of the resulting color was assessed at a wavelength of 450 nm using an enzyme-linked immunosorbent assay reader. The concentrations of COX-2 and PGE2 were determined by comparing the optical density values with those of the standard concentration curve.[16]

Assessment of anti-inflammatory activity

The mice with an average weight of 25-30 g were divided into 6 groups (n = 5). Saline group: mice were pre-treated with 10 mL/kg of sterile saline solution orally and did not induce inflammation. Carrageenan 1% group: mice were inflamed by injecting 1% carrageenan (50 µL) and received no group. Carr + Indomethacin group: mice were inflamed by injecting carrageenan (Carr) 1% (50 µL) and orally administered indomethacin (10 mg/kg). Carr + ($EHCW_{200}$, $EHCW_{250}$, and $EHCW_{300}$) groups: mice were inflamed by injecting carrageenan (Carr) 1% (50 µL) and orally administered EHCW (200, 250, and 300 mg/kg, respectively).

Paw edema induced by carrageenan

The anti-inflammatory efficacy of EHCW was assessed through the carrageenan-induced paw edema test in the hind paws of mice. The procedure, with slight adjustments, followed the methodology outlined by Almasarwah *et al*.[17] Before drug administration, mice that had fasted overnight were provided access to water, and the initial volume of the right hind paw was determined. Subsequently, 50 µL of carrageenan (1% w/v in physiological saline, 0.05 mL) was injected into the sub-plantar region of the right hind paw. Carrageenan injection occurred 30 min after the administration of the extract (at doses of 200, 250, and 300 mg/kg), vehicle (saline, 10 mL/kg), or the standard drug (indomethacin, 10 mg/kg) for the respective mouse groups. Paw edema was quantified in millimeters, representing the linear circumference of the paw, as per the method outlined by Elsheemy *et al*., using a Mitutoyo digital caliper (Japan) at intervals of 0, 1, 2, 3, 4, and 5 hours following carrageenan injection.[18] The percentage inhibition of edema (PEI) was calculated in comparison to the control group as follows:

$$\text{PEI (\%)} = \frac{\text{PEC} - \text{PET}}{\text{PEC}} \times 100$$

while: PEC paw edema in the control group; PET paw edema in the test group.

Malondialdehyde (MDA) assay

Carrageenan injection induced the generation of malondialdehyde (MDA), which was evaluated utilizing the thiobarbituric acid reactive substances (TBARS) assay, following the protocol outlined by Chang *et al.*[14] MDA underwent a reaction with TBARS at elevated temperatures, resulting in the formation of a red-colored TBARS complex. The absorbance of TBARS was measured at a wavelength of 532 nm.

TNF- α, IL-1β, and IL-6 testing

The quantification of IL-1β was carried out utilizing an enzyme-linked immunosorbent assay (ELISA) method in combination with an immune adsorption assay, following the procedure outlined by Chang *et al*.[14] In this process, IL-1β capture antibodies were immobilized in individual wells of a 96-well plate and allowed to adhere overnight. The subsequent day, a biotin-labeled antibody was added to the plate after incubating tissue samples or standard antigens. Streptavidin was then introduced, leading to a color shift from purple to yellow, which was quantified at a wavelength of 450 nm. A similar method was employed for the detection of IL-6 and TNF-α. The concentration of TNF-α, IL-6, and IL-1β in each sample was expressed as ng/mg.

Antipyretic activity

The assessment of antipyretic activity was conducted employing a yeast-induced fever model in laboratory animals, following the procedure delineated by Onyegbule *et al.*[19] The mice were categorized into six groups (n = 5 in each group). In the saline group, mice were orally administered a pre-group of 10 mL/kg sterile saline solution and were not subjected to fever induction. In the yeast group, mice were induced with fever by the injection of yeast (10 mL/kg) and were not provided with any subsequent group. Yeast + Paracetamol group: mice were inflamed by injecting yeast (10 mL/kg) and orally administered paracetamol (150 mg/kg). Yeast + ($EHCW_{200}$, $EHCW_{250}$, and $EHCW_{300}$) groups: mice were inflamed by injecting carrageenan yeast (10 mL/kg) and orally administered EHCW (200, 250, and 300 mg/kg, respectively).

To induce a state of high fever, a subcutaneous injection of a 20% yeast suspension at a dosage of 10 mL/kg of body weight was administered. Before the experiment, the animals underwent an overnight fast, with access to water ad libitum. The baseline rectal temperatures of the animals were measured using a TP101 thermometer (China). After 18 h following the subcutaneous injection, animals displaying a rectal temperature increase within the range of 0.3 - 0.5°C were selected for the assessment of antipyretic activity. Oral administration of EHCW extract (at doses of 200, 250, and 300 mg/kg) was performed. Paracetamol (administered orally at a dose of 150 mg/kg) served as the reference drug, while the control group was provided with saline (10 mL/kg). Rectal temperatures were monitored at 1, 2, and 3-hour intervals post-group, and the percentage of fever reduction (PFR) was calculated using the formula:

$$\text{PFR (\%)} = \frac{\text{B} - C_n}{\text{B} - \text{A}}\ x\ 100$$

with B is the post-fever temperature; C_n is the temperature after 1, 2, 3 h and A is the normal body temperature.

Statistical analysis

Statistical analysis was performed using Statgraphics Centurion software version XIX. The results are expressed as mean values with accompanying standard deviations (SD). Statistical significance was assessed through one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to compare intergroup variations. Statistical significance was established at $p < 0.05$. The data obtained from the analysis were tabulated and graphically represented.

Results and Discussion

Phytochemical, total phenolic (TPC), total flavonoid (TFC) and phytochemical of ethanol leaf extract of *H. capitellata*

The presence of plant chemicals in ethanol leaf extract of *H. capitellata* was demonstrated through positive chemical reactions (Table 1), as indicated by notable color changes. Flavonoids, alkaloids, and polyphenols were the most abundant compounds among the screened plant types. Flavonoids exhibited particularly strong positive reactions with distinct color changes. Alkaloids and polyphenols were the next prominent compound group present in

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

EHCW. Additionally, tannins, saponins, glycosides, and terpenoids were also detected. However, steroids were absent in EHCW.

The ethanol extract from *H. capitellata* leaves underwent phytochemical screening, revealing the presence of various secondary metabolites, including alkaloids, flavonoids, polyphenols, and more. These identified chemical compounds within EHCW have known medicinal significance. Alkaloids derived from medicinal plants have demonstrated a range of biological activities, such as anti-inflammatory, antipyretic, antimicrobial, cytotoxic, antispasmodic, and other pharmacological effects. Tannins, on the other hand, exhibit antibacterial, anti-tumor, and antiviral properties by precipitating microbial proteins, rendering nutritional proteins inaccessible for microbial utilization. Additionally, glycosides find application in the group of congestive heart failure and cardiac arrhythmias by inhibiting the $Na^+/K+$ pump, resulting in increased Ca^{2+} levels. Consequently, this elevated Ca^{2+} availability facilitates cardiac muscle contraction, contributing to the restoration of cardiac output and the reduction of cardiac stress.[10] The plant chemical compounds identified in EHCW may contribute to the biological activities of *H. capitellata* and explain its traditional medicinal use among indigenous populations.

The TPC and TFC content in EHCW

The results in Table 2 showed that EHCW had a total polyphenol content of 68.98 ± 2.49 mg GAE/g of extract, while the total flavonoid content was 42.77 ± 3.16 mg QE/g of extract.

Plant polyphenols play a pivotal role in neutralizing free radicals and functioning as primary antioxidants. Consequently, assessing the polyphenol content in plant extracts is a logical step. Polyphenolic compounds are characterized by aromatic benzene rings adorned with hydroxyl groups, including their functional derivatives. These compounds possess the capability to intercept free radicals and can also chelate metal ions that might otherwise catalyze the generation of reactive oxygen species (ROS) and promote lipid peroxidation. Among polyphenols, flavonoids hold particular importance due to their contribution to an organism's defense against diseases. The antioxidant effectiveness of flavonoids hinges on factors such as molecular structure, hydroxyl group placement, and other chemical characteristics.[10] In our investigation, the total polyphenol and flavonoid content in EHCW were measured at 68.98 ± 2.49 mg GAE/g of extract and 42.77 ± 3.16 mg QE/g of extract, respectively (Table 2). These values are on par with or slightly exceed the total flavonoid content found in other medicinal plants. For instance, the flavonoid content in *Xylopia aethiopica* seed extract was 35 mg QE/g of extract,[20] and in *Biophytum sensitivum* leaf extract, it was 22 mg QE/g of extract.[21] Consequently, it is plausible to infer that polyphenols and flavonoids within *H. capitellata* may act synergistically with other chemical constituents, underscoring its medicinal significance.

Assessment of analgesic activity

Writhing test provoked by acetic acid.

The outcomes of the acetic acid-induced writhing test in mice are outlined in Table 3. In comparison to the untreated control group, both the ethanol leaf extract of *H. capitellata* and aspirin (used as reference) significantly reduced the number of writhing episodes in mice subjected to intraperitoneal acetic acid injection ($p < 0.05$). The percentage inhibition of writhing (PIW) by EHCW exhibited a dose-dependent increase (46.62%, 60.09%, and 70.66%) ($p < 0.05$) with the tested doses (200, 250, and 300 mg/kg, respectively). The maximum inhibition of writhing, 70.66%, was achieved with a 300 mg/kg dose of EHCW. The inhibitory effect of EHCW at a dose of 300 mg/kg (70.66%) was comparable to that of aspirin at 10 mg/kg (75.47%).

Table 1: Phytochemical screening of ethanol extracts of *H. capitellata* leaves

Phytochemicals	Present in EHCW	Phytochemicals	Present in EHCW
Alkaloid	+	Glycoside	+
Tannin	+	Steroid	-
Saponin	+	Terpenoid	+
Polyphenol	+	Flavonoid	+

Presence of phytochemicals in EHCW: "+" present, "-" absent

Table 2: Total polyphenol and flavonoid content of ethanol leaf extract of *H. capitellata*

Extract	Total polyphenol content (mg GAE/g of extract)	Total flavonoid content (mg QE/g of extract)
EHCW	68.98 ± 2.49	42.77 ± 3.16

GAE = gallic acid equivalent, QE = quercetin equivalent

Table 3: Analgesic effect of EHCW on acetic acid-induced writhing pain

Parameters	Saline group	Aspirin group	$EHCW_{200}$ group	$EHCW_{250}$ group	$EHCW_{300}$ group
Number of times writhing (times)	46.22 ± 0.18^a	11.34 ± 0.17^e	24.67 ± 0.18^d	18.45 ± 0.14^c	13.56 ± 0.22^b
PIW (%)	0.00 ± 0.00^a	75.47 ± 0.29^e	46.62 ± 0.28^b	60.09 ± 0.18^c	70.66 ± 0.39^d

Values are expressed as Mean ± SD, letters a, b, c, d, and e represent the difference between groups ($p < 0.05$).

Table 4: Analgesic effects of EHCW on formalin-induced pain

Experiment phase	Paw licking time (seconds)					
	Saline group	Aspirin group	Tramadol group	$EHCW_{200}$ group	$EHCW_{250}$ group	$EHCW_{300}$ group
Early phase	19.93 ± 0.34^a	32.19 ± 0.23^f	29.36 ± 0.21^e	23.39 ± 0.22^b	25.48 ± 0.27^c	28.84 ± 0.23^d
Later phase	17.78 ± 0.27^a	29.65 ± 0.25^e	34.55 ± 0.27^e	26.72 ± 0.21^b	29.43 ± 0.35^c	31.77 ± 0.28^d

Values are expressed as Mean ± SD, the letters a, b, c, d, e, and f represent the difference between the groups ($p < 0.05$).

Table 5: Effect of EHCW on the plasma concentrations of COX-2 and PGE2 in the formalin test

Parameters	Time (min)	Saline group	Tramadol group	$EHCW_{200}$ group	$EHCW_{250}$ group	$EHCW_{300}$ group
COX-2 levels (ng/mL)	0	46.22 ± 0.18^a	11.34 ± 0.17^e	24.67 ± 0.18^d	18.45 ± 0.14^c	13.56 ± 0.22^b
	30	6.44 ± 0.18^c	4.58 ± 0.27^a	5.05 ± 0.24^b	4.96 ± 0.19^b	4.77 ± 0.16^{ab}
PGE2 levels (ng/mL)	0	0.89 ± 0.07^d	0.51 ± 0.08^a	0.72 ± 0.08^c	0.65 ± 0.09^{bc}	0.61 ± 0.08^{ab}
	30	0.99 ± 0.08^d	0.41 ± 0.06^a	0.61 ± 0.09^c	0.54 ± 0.07^{bc}	0.47 ± 0.07^{ab}

Values are expressed as Mean ± SD, letters a, b, c, d represent the difference between groups ($p < 0.05$).

The acetic acid-induced writhing test was conducted to assess the peripheral analgesic potential of the test compound. Acetic acid is known to induce pain and inflammation by increasing capillary permeability and releasing pain-inducing substances at nerve endings. Peripheral analgesic effects typically involve the activation of intermediaries that inhibit cyclooxygenase, lipoxygenase, and other pro-inflammatory mediators or suppress pain responses through peripheral receptor intermediaries.[22] In this study, EHCW demonstrated a significant reduction ($p < 0.05$) in the total number of abdominal constrictions, suggesting an enhancement in the release of pain-inhibiting intermediaries. Pain perception is initiated by the local inflammatory response, which involves the release of endogenous substances and pain-inhibiting intermediaries like arachidonic acid via prostaglandin synthesis. Prostaglandin products at the site of tissue damage contribute to inflammation and pain by increasing capillary permeability. [22] The phytochemical screening of EHCW confirmed the presence of various plant chemical compounds such as alkaloids, flavonoids, and polyphenols, among others. Consequently, these plant chemical compounds may induce analgesic, anti-inflammatory, and anti-infective effects by inhibiting the release of pro-inflammatory intermediaries like serotonin, histamine, and prostaglandin, as well as by reducing blood flow.

Formalin test

Ethanol leaf extract of *H. capitellata* demonstrated dose-dependent effects in the two phases of the formalin-induced pain test. The results in Table 4 showed the licking time (seconds) for each group in the different phases as follows: Early phase (aspirin group > tramadol group > $EHCW_{300}$ group > saline group) ($p < 0.05$). In this phase, the licking time significantly increased with group of EHCW at doses of 200, 250, and 300 mg/kg. Also, in the early phase, the response time in the aspirin-treated group was significantly higher than in the tramadol group ($p < 0.05$). In contrast, in the late phase (tramadol group > aspirin group > $EHCW_{300}$ group > saline group) ($p < 0.05$), the response time in the aspirin and tramadol groups showed reversed results, while the licking time in the EHCW groups continued to increase significantly compared to the saline control group ($p < 0.05$). The pain inhibition ratio (PIF) (Figure 1) also exhibited similar changes, with the percentage of pain inhibition by EHCW in the groups at doses of 200, 250, and 300 mg/kg (14.81% and 33.45%, 21.79% and 39.59%, 30.9% and 44.03%, respectively) significantly increasing compared to the saline group (0%) ($p < 0.05$).

The formalin test is a chronic pain assessment that exhibits a biphasic response pattern. The early phase, known as the neurogenic pain response, manifests within the initial 5 min following formalin injection. In contrast, the late phase, characterized as the inflammatory pain response, emerges approximately 20 to 30 min post-injection. Central-acting drugs, such as tramadol, are capable of inhibiting both phases, with particular effectiveness in phase 2. On the other hand, peripheral-acting drugs like aspirin are primarily efficient at inhibiting pain during the early phase.[14] This peripheral analgesic activity is attributed to the suppression of the production and release of specific endogenous inflammatory mediators within the pain perception pathway, particularly the prostaglandin pathway. This mechanism involves the blockade of the cyclooxygenase enzyme pathway and the reduction of the sensitivity of peripheral pain-inducing substances.[1] The central nervous system, including the brain and spinal cord, plays pivotal roles in central pain mechanisms. The dorsal region of the spinal cord contains various substances like substance P, endogenous opioids, somatostatin, and other inhibitory hormones that serve as targets for pain and inflammation.[22] Analgesic drugs with addictive properties, such as tramadol, exhibit effects against both peripheral and central pain, while nonsteroidal anti-inflammatory drugs like aspirin primarily target peripheral pain. The group using EHCW at doses of 200, 250, and 300 mg/kg demonstrated a reduction in pain responses during both phases of formalin-induced pain. These findings suggest that EHCW may possess analgesic effects akin to those of addictive analgesics and nonsteroidal anti-inflammatory drugs.

Effect of EHCW on the plasma levels of COX-2 and PGE2

The data showed significantly lower levels of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) expression in serum at 30 min in the formalin test in the EHCW300 and tramadol groups compared to the saline group ($p < 0.05$). The expression levels (ng/mL) of COX-2 were demonstrated in the saline group (6.44 ± 0.18), $EHCW_{300}$ group (4.77 ± 0.16), and tramadol group (4.58 ± 0.27), respectively. The levels of PGE2 were demonstrated in the saline group (0.99 ± 0.08), $EHCW_{300}$ group (0.47 ± 0.07), and tramadol group (0.41 ± 0.06), respectively (Table 5). The test results confirmed that the group methods with EHCW and tramadol effectively inhibited the COX-2 concentration and reduced PGE2 production to achieve analgesic effects.

The formalin-induced pain test exhibits a two-phase response pattern. In the early phase of inflammatory pain, substances such as histamine, bradykinin, and 5-hydroxytryptamine (5-HT) are released. Subsequently, during the late phase, there is an increased production of TNF-α, IL-1β, COX-2, and PGE2, which can intensify the severity of inflammatory pain. COX-2 expression reaches its peak in the late phase of the formalin-induced pain test, leading to an elevated concentration of prostaglandin in both inflammatory and pain responses. COX-2 interacts with arachidonic acid to generate prostaglandin, which in turn triggers a sensation of pain. Tramadol exerts its analgesic effect by inhibiting COX-2, consequently reducing the prostaglandin concentration.[23] The serum ELISA results indicate a substantial inhibition of COX-2 in the tramadol and EHCW group groups at doses of 200, 250, and 300 mg/kg. Furthermore, the levels of PGE2 in the tramadol and EHCW groups were significantly lower than those in the saline group group ($p < 0.05$).

Assessment of anti-inflammatory

Effect of EHCW on carrageenan-induced mouse paw edema

The inhibitory effects comparison after group with EHCW and indomethacin on carrageenan-induced paw edema is presented in Table 6 and Figure 2. Subcutaneous injection of 1% carrageenan resulted in increased paw size in mice due to edema. The maximum foot circumference was observed after 5 h of the experiment, especially in the carrageenan untreated group (68.07 ± 0.2 mm), indicating acute inflammation of the foot.

Table 6: Effects of EHCW against carrageenan induced paw edema model in mice

Experimental group	Circumference paw (mm)				
	1 h	2 h	3 h	4 h	5 h
Saline group	22.36 ± 0.12^{a}	22.36 ± 0.12^{d}	22.36 ± 0.12^{d}	22.36 ± 0.12^{a}	22.36 ± 0.12^{e}
Carrageenan group	30.86 ± 0.32^{f}	36.94 ± 0.16^{e}	44.17 ± 0.17^{e}	55.03 ± 0.22^{f}	68.07 ± 0.2^{f}
Carr + Indomethacin group	23.28 ± 0.18^{b}	21.03 ± 0.27^{a}	20.09 ± 0.32^{a}	19.01 ± 0.51^{a}	18.09 ± 0.19^{a}
Carr + $EHCW_{200}$ group	24.92 ± 0.22^{e}	22.72 ± 0.16^{d}	21.81 ± 0.48^{c}	20.96 ± 0.26^{d}	19.58 ± 0.34^{d}
Carr + $EHCW_{250}$ group	24.59 ± 0.26^{d}	22.06 ± 0.3^{c}	21.06 ± 0.36^{b}	20.11 ± 0.32^{c}	19.07 ± 0.21^{c}
Carr + $EHCW_{300}$ group	23.82 ± 0.14^{c}	21.65 ± 0.29^{b}	20.67 ± 0.35^{b}	19.62 ± 0.35^{b}	18.54 ± 0.25^{b}

Values are expressed as Mean ± SD, letters a, b, c, d, e represent the difference between groups ($p < 0.05$).

Experimental data from the groups treated with EHCW at doses of 200, 250, and 300 mg/kg at the 5-hour time point showed a significant reduction in foot edema size (19.58 ± 0.34 mm, 19.07 ± 0.21 mm, and 18.54 ± 0.25 mm, respectively) ($p < 0.05$) compared to the carrageenan group, equivalent to the standard drug indomethacin (18.09 ± 0.19 mm). The current study also demonstrated the highest inhibition of foot edema at 72.76% in the $EHCW_{300}$ group, significantly different from the $EHCW_{200}$ and $EHCW_{250}$ groups (71.73% and 71.98%, respectively) ($p < 0.05$), which was nearly equivalent to the standard drug indomethacin (73.42%) after 5 h of the experiment. EHCW (at a dose of 300 mg/kg body weight) exhibited significant anti-inflammatory activity ($p < 0.05$) compared to saline and indomethacin.

The carrageenan-induced paw edema model is a well-established method for assessing the anti-inflammatory properties of novel compounds. In this model, inflammation progresses through two primary stages. The initial stage, occurring within the first hour after carrageenan administration, involves the release of various mediators, including histamine, leukotrienes, kinins, serotonin, and cyclooxygenase. Subsequently, the late phase ensues, characterized by the production of prostaglandins, bradykinin, proteases, lysosomes, and neutrophil infiltration. The late phase is particularly sensitive to the effects of clinically effective anti-inflammatory drugs.[24] EHCW, administered at doses of 200, 250, and 300 mg/kg, led to a significant reduction in paw size from 1 to 5 hours, demonstrating a dose-dependent anti-inflammatory response comparable to that of the standard drug indomethacin. This observation suggests that EHCW may exert its acute anti-inflammatory effect through multiple mechanisms, potentially involving the inhibition of enzymes such as cyclooxygenase and lipoxygenase, as well as the modulation of the synthesis, release, and activity of the aforementioned inflammatory mediators.

Effect of EHCW on MDA, TNF- α, IL-1β and IL-6 levels

Malondialdehyde (MDA) is the end product generated from the breakdown of arachidonic acid and large polyunsaturated fatty acids through enzymatic or non-enzymatic processes. Excessive production of MDA is associated with various pathological conditions. In this study, MDA levels were used to indicate the process of lipid peroxidation. As shown in Table 7, MDA levels significantly increased in the carrageenan group (2.84 ± 0.14 nmol/mg) ($p < 0.05$). However, group with 300 mg/kg EHCW (1.78 ± 0.23 nmol/mg) and 10 mg/kg indomethacin (1.35 ± 0.15 nmol/mg) demonstrated significant inhibition of MDA elevation ($p < 0.05$). Lipid peroxidation is a process in which oxidative agents such as free radicals attack lipids containing carbon-carbon double bonds, particularly polyunsaturated fatty acids (PUFAs), leading to the abstraction of hydrogen from carbon and the insertion of oxygen into lipid peroxyl and hydroperoxide radicals. MDA is an aldehyde formed as a result of toxic stress in cells and forms covalent adducts with proteins called end products of lipid peroxidation. MDA is generated in prostaglandin synthesis, and the inflammatory response leads to MDA accumulation.[25] In this study, EHCW and indomethacin significantly reduced MDA levels, suggesting that EHCW is effective in counteracting inflammatory agents in mice.

Cytokines are small proteins secreted by cells that affect cell interaction and communication. TNF-α, IL-1β, and IL-6 are pro-inflammatory cytokines primarily produced by activated macrophages and participate in regulating increased inflammatory responses. The results in Table 7 show a significant increase in serum levels of TNF-α, IL-1β, and IL-6 in the carrageenan-induced foot edema group ($p < 0.05$). Group with EHCW (300 mg/kg) significantly reduced the concentrations of TNF-α, IL-1β, and IL-6 (184.68 ± 23.51, 369.76 ± 22.05, and 25.46 ± 1.15 pg/mL, respectively) compared to the carrageenan group (289.55 ± 30.54, 629.46 ± 27.27, and 66.77 ± 1.22 pg/mL, respectively) ($p < 0.05$), which was comparable to the Carr + Indomethacin group (165.76 ± 33.69, 342.54 ± 26.03, and 21.82 ± 1.09 pg/mL). IL-1β, IL-6, and TNF-α are pro-inflammatory cytokines associated with the pathological process of pain, and they are involved in the recruitment of neutrophils in the carrageenan-induced inflammatory response. IL-1β is released by monocytes and macrophages as well as by endothelial and epithelial cells in response to infection, invasion, and inflammation. IL-6 plays an activating role in nerve injury and inflammation. TNF-α is a key cytokine in the inflammatory process, acting on different signaling pathways through TNFR1 and TNFR2 receptors to regulate apoptotic pathways, activate NF-kB and protein kinase inflammatory reactions, and activate stress-activated protein kinase (SAPK).[23] In this study, EHCW and indomethacin showed significant anti-inflammatory effects by reducing the concentrations of TNF-α, IL-1β, and IL-6.

Antipyretic activity

Subcutaneous injection of 10 mL/kg of 20% yeast extract solution was used to induce hyperthermia in mice. The results in Table 8 and Figure 3 demonstrate that the hyperthermia caused by yeast extract in mice was reduced after group with 200, 250, and 300 mg/kg of EHCW at 1, 2, and 3 h, respectively, with significant effects ($p < 0.05$). The initial rectal temperature of 36.22 - 36.27°C increased to 39.45°C and 39.85°C ($p < 0.05$) after yeast extract administration.

Table 7: Effect of EHCW on MDA, TNF-*α*, IL-1*β*, and IL-6 level in the edema paws

Parameters	Saline group	Carr group	Carr + Indomethacin group	Carr + $EHCW_{200}$ group	Carr + $EHCW_{250}$ group	Carr + $EHCW_{300}$ group
MDA (nmol/mg protein)	1.24 ± 0.09^{a}	2.84 ± 0.14^{e}	1.46 ± 0.08^{ab}	23.39 ± 0.22^{d}	2.07 ± 0.23^{cd}	1.78 ± 0.23^{bc}
TNF-*α* (pg/mL)	139.51 ± 18.93^{a}	289.55 ± 30.54^{d}	165.76 ± 33.69^{ab}	229.85 ± 28.31^{c}	225.35 ± 27.21^{c}	184.68 ± 23.51^{b}
IL-1*β* (pg/mL)	271.29 ± 23.01^{a}	629.46 ± 27.27^{d}	342.54 ± 26.03^{b}	438.46 ± 23.52^{c}	419.63 ± 19.13^{c}	369.76 ± 22.05^{b}
IL-6 (pg/mL)	17.76 ± 1.08^{a}	66.77 ± 1.22^{f}	21.82 ± 1.09^{b}	34.29 ± 1.25^{e}	31.33 ± 1.16^{d}	25.46 ± 1.15^{c}

Values are expressed as Mean ± SD, letters a, b, c, d, e, and f represent the difference between groups ($p < 0.05$).

Table 8: Effect of EHCW at therapeutic doses of 200, 250, and 300 mg/kg on yeast-induced fever

Experimental group	Initial (^{o}C)	Fever (^{o}C)	1 h (^{o}C)	2 h (^{o}C)	3 h (^{o}C)
Saline group	36.22 ± 0.22^{a}	36.23 ± 0.21^{a}	36.25 ± 0.2^{a}	36.27 ± 0.2^{a}	36.23 ± 0.21^{a}
Yeast group	36.17 ± 0.19^{a}	39.45 ± 0.17^{bc}	39.68 ± 0.18^{e}	39.78 ± 0.12^{d}	39.85 ± 0.11^{d}
Yeast + Paracetamol group	36.19 ± 0.21^{a}	39.34 ± 0.11^{b}	38.41 ± 0.14^{b}	37.71 ± 0.19^{b}	36.84 ± 0.18^{b}
Yeast + $EHCW_{200}$ group	36.23 ± 0.23^{a}	39.71 ± 0.14^{d}	38.81 ± 0.13^{d}	38.26 ± 0.15^{c}	37.62 ± 0.17^{c}
Yeast + $EHCW_{250}$ group	36.27 ± 0.13^{a}	39.59 ± 0.18^{cd}	38.69 ± 0.14^{cd}	38.12 ± 0.15^{c}	37.43 ± 0.16^{c}
Yeast + $EHCW_{300}$ group	36.25 ± 0.11^{a}	39.46 ± 0.11^{bc}	38.57 ± 0.16^{bc}	37.82 ± 0.14^{b}	36.98 ± 0.19^{b}

Values are expressed as Mean ± SD, letters a, b, c, d, e represent the difference between groups ($p < 0.05$).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

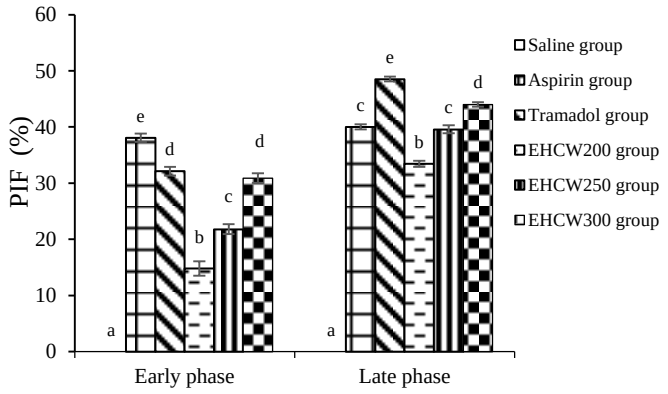

Figure 1: The percentage of pain inhibition (PIF) of EHCW in in the formalin test

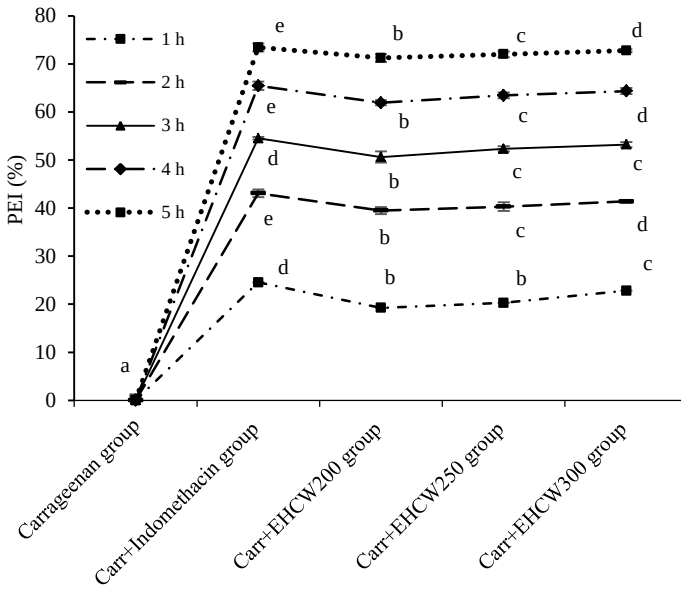

Figure 2: The percentage inhibition of edema (PEI) of EHCW in anti carrageenan-induced paw oedema

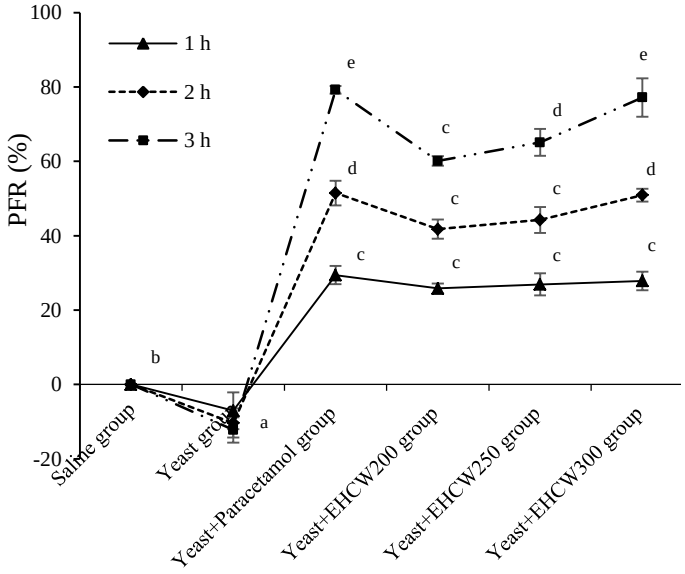

Figure 3: The percentage of fever reduction (PFR) of EHCW in anti yeast-induced fever

However, the temperature returned to 36.98°C after 3 h of group with 300 mg/kg of EHCW, which was comparable to the temperature achieved with the standard drug indomethacin (36.84 ± 0.18°C) ($p > 0.05$) (Table 8). The percent fever reduction (PFR) also significantly increased after 3 h of group with EHCW and paracetamol. The fever inhibition percentage of EHCW at 300 mg/kg reached 77.19%, which was close to paracetamol's 79.24% ($p > 0.05$) (Figure 3).

Fever induction in animal models involves various exogenous substances, including bacterial endotoxins and infections, acting as exogenous pyrogens. These pyrogens trigger the production of pro-inflammatory cytokines, leading to the release of local prostaglandins (PGs). This process results in the resetting of the hypothalamic set-point, which governs body temperature regulation by balancing heat production and loss. Non-steroidal anti-inflammatory drugs are known to inhibit prostaglandin synthesis in the hypothalamic set-point, displaying antipyretic effects.[26] In the present study, EHCW exhibited a significant antipyretic effect, yielding a fever reduction percentage (PFR) comparable to that of the standard drug in lowering body temperature. This suggests that EHCW may exert its antipyretic effects by inhibiting prostaglandin synthesis in the hypothalamic set-point, akin to the action of paracetamol. These pharmacological findings provide support for the traditional belief in the antipyretic potential of *H. capitellata* leaves.

Conclusion

The study evaluated the analgesic, anti-inflammatory, and antipyretic effects of ethanol extract from *H. capitellata* leaves in animal models. The results demonstrated that ethanol leaf extract of *H. capitellata* effectively reduced pain by enhancing pain inhibition in Swiss mice in the acetic acid writhing test and formalin-induced pain test. Based on ELISA results, ethanol leaf extract of *H. capitellata* alleviated pain by effectively reducing the concentration of PGE2 in the serum through COX-2 inhibition. The anti-inflammatory potential of *H. capitellata* leaves was also demonstrated through the anti-inflammatory effect of ethanol leaf extract of *H. capitellata* (at all doses), which reduced paw circumference, decreased MDA levels, and reduced cytokines (TNF-α, IL-1β, and IL-6) in the serum of mice with carrageenan-induced inflammation. The extract from *H. capitellata* leaves also significantly alleviated fever caused by yeast extract in mice by reducing body temperature and increasing the fever inhibition rate of the extract through the inhibition of prostaglandin E2 synthesis. These findings support the use of *H. capitellata* as a potential medicine for inflammation, pain relief, and fever reduction in the future.

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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