Trop J Nat Prod Res, February 2025; 9(2): 447 - 453 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

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

Original Research Article

Evaluation of Membrane Stabilizing, Thrombolytic, Anti-Diarrheal and Antipyretic Activities of Ethanolic Extracts of *Hoya Parasitica* variegata and *Crotalaria pallida* Aiton

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ARTICLE INFO

Article history:
Received 29 September 2025
Revised 08 October 2025
Accepted 28 October 2025
Published online 01 March 2025

ABSTRACT

Hoya parasitica Variegata (Apocynaceae) and *Crotalaria pallida* Aiton (Fabaceae)are traditionally used to treat pain, fever, and urinary disorders, but scientific1reports are limited or lacking on their antipyretic, antidiarrheal, thrombolytic, and membrane-stabilizing activities. The study evaluated the membrane-stabilizing, thrombolytic, anti-diarrheal, and antipyretic activities of crude extracts from *H. parasitica* and *C. pallida* using various animal models. In the *in vitro* thrombolytic test, assessing clot lysis, the extracts of *H. parasitica* and *C. pallida* showed thrombolytic activity with clot lysis of 75.93% and 73.64%, respectively, compared to streptokinase, which achieved 85.17% clot lysis. In the hypotonic buffer-induced hemolysis test, the ethanolic extracts of *H. parasitica* and *C. pallida* inhibited hemolysis of red blood cells (RBC) by 64.53%and 68.23%, respectively, which is close to the inhibition rate of acetyl salicylic acid (79.68%). Similarly, in the heat-induced hemolysis test, the extracts inhibited RBC hemolysis by 62.89% and 68.14%, compared to the 74.49% inhibition observed with acetyl salicylic acid. In the castor oil-induced diarrhea, the ethanolic extracts of *H. parasitica* and *C. pallida* leaves at doses of 100,150, and 200 mg/kg body weight significantly ($P<0.05$) reduced the number of diarrheal faeces compared to the negative control. Additionally, in the brewer's yeast-induced pyrexia test, these extracts at the same doses elevated temperatures in mice in a dose-dependent manner, with significant ($P<0.05$) reductions observed at the 21st, 22nd, and 23rd hours of treatment. These results indicate that the ethanol extracts of *H. parasitica* and *C. pallida* exhibit notable thrombolytic, membrane-stabilizing, anti-diarrheal, and antipyretic properties.

Keywords: Thrombolytic; membrane stabilizing; anti-diarrheal; antipyretic; *Hoya parasitica* and *Crotalaria pallida*

Introduction

Traditional medicine is defined as a body of theory and expertise that is used to diagnose, prevent, and treat disease. This could be based on previous experiences and perceptions conceded from one generation to others.[1] Essentially, traditional medicine is expanding its boundaries, and plants remain a unique source of structurally essential components that contribute to the development of novel medications.[2] Herbalists can get the therapeutic effects of various plants by performing different tests of plants and after a series oftrial and error they can get the desired results. Thus the list of medicinal herbs is enriching day by day. Many medications utilized in modern medicine are derived from plants and were first identified through native people's ancient practices.[3] Natural sources as medicines are gaining popularity as a result of their low toxicity or lack of toxicity, complete biodegradability, availability from natural sources, and, in most cases, inexpensive cost as compared to molecules produced through comprehensive synthetic chemistry.[4]

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Citation: Bulbul IJ, Shanta AP, Mahmud MP, Jahan N, Ahmed Z. Evaluation of membrane stabilizing, thrombolytic, anti-diarrheal and antipyretic activities of ethanolic extracts of *Hoya parasitica* Variegata and *Crotalaria pallida* Aiton. Trop J Nat Prod Res. 2025; 9(2):447 - 453 https://doi.org/10.26538/tjnpr/v9i2.5

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Several persons in Bangladesh, because of their economy and/or tradition, rely on medicinal plant resources. Bangladesh has had several tribes of aboriginal people using medicinal herbs to heal different illnesses for many years. The tribes' younger generations have begun to acquire the culture as well as legacy.These tribes have amassed a vast amount of information, notably about the medicinal characteristics of numerous plant species, due to their decades of living in forest and hill track regions, [5] and they continue to employ a variety of medicinal herbs. However, there are little records or academic proof of the indigenous people of Bangladesh's ancient use of those herbs. Considering the importance of natural sources and the perspective of Bangladesh, two different locally available medicinal plants of Bangladesh, *Hoya parasitica* Roxb and *Crotalaria pallida* Aiton were selected for this study for their membrane stabilizing, thrombolytic, anti-diarrheal, and antipyretic activities.

The common names for *Hoya parasitica* Roxb. (Family: Apocynaceae) are Fessyagach (Chakma), Chera pata (Rema-Kalenga) which is traditionally used to treat urinary tract disorders, pain and fever, jaundice, bronchitis and diabetes.[6] Sadhu et al. (2008) reported 15-bulnesolic acid, hoyasterone, 1-(4-hydroxy-3-methoxyp henyl)-1-methoxypropan-2-ol, dihydrocanaric acid from *H. parasitica* among which dihydrocanaric acid was showed to have growth inhibitory action against SW480 and HeLa cells.[7] According to a previous study, this plant contains three steroidal glycosides, identified as 3*β*, 4*α*-dihydroxy-5*β*-spirost-(25), lupeol, triterpenic 3, 4-seco acid 3, 4-secolup-20(29)-en-3-oic acid, and lupenone from stem.[8] O-*α*-L-arabinopyranoside 27-en-1*β*-yl, 3-hydroxy-5*β*-spirost-25(27) L-rhamnopyranosyl-(1→2)-en-1*β*-ylO-*α*-L-*β*-D-xylopyranoside, (23S,24S,25S)-3*β*,4*α*,23,24-tetrahydroxy-5*β*-spirostan-1*β*-yl O-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-fucopyranoside,[9,10] two pregnane

glycosides from the leaf extract, called parasiticoside A and B, and foligenin and calogenin [11] from *H. parasitica*. Sarkar *et al*. (2023) revealed *H. parasitica* to have its antioxidant, analgesic, antipyretic and antidiarrheal properties. Besides, *in-silico* study of hoyasterone proved that it is a good cyclooxygenase enzyme inhibitor.[12]

Crotalaria pallida Aiton (Fabaceae) is called to the indigenous people of Bangladesh as Kudug Jhunjhuni (Chakma) and has traditionally been used for urinary issues, painful joint swelling, fever, and as a vermifuge. Earlier studies revealed lectins,[13] antimicrobial peptides,[14] three pterocarpanoids ascrotafurans A, B, and C,[15] anti-inflammatory flavonoids and pterocarpanoid like, isoflavone as 5,7,40-trihydroxy-20–methoxyisoflavone, apigenin, hydroxygenistein1, daidzein, hydroxydaidzein[16] estrogenic and mutagenic stigmasterol,[17] homo isoflavonoids with rare skeletons, alkaloids asusaramine-N-oxide, cropallins A and B, cytotoxicity and anti-inflammatory cropalliflavones A, B and C,[18] anti-tyrosinase crotalariapallins A, B and C[19] from different parts of *C. pallida.* In an earlier study, nine saturated and unsaturated fatty acids were isolated by gas-liquid chromatography from the leaf oil of *C. pallida* among which linolenic acid and palmitic acid showed promising antimicrobial activities against Gm +ve bacteria, *Bacillus subtilis*, and Gram -ve bacteria, *Acinetobacter junii* and *Escherichia coli* .[20]
Few reports discovered the antipyretic and antidiarrheal activities of *H.parasitica* but no report for its thrombolytic and membrane stabilizing activities. Though some of the isolated phytoconstituents from *C. pallida* were reported for their antimicrobial, anti-inflammatory, estrogenic, mutagenic, and cytotoxic activities but there are no reports for its thrombolytic, antipyretic,membrane stabilizing, and antidiarrheal activities.
Thus, the current study set out to examine the thrombolytic, antipyretic,membrane-stabilizing, and antidiarrheal characteristics of *C. pallida* and *H.parasitica*.

Materials and Methods

Plant collection
H parasitica and *C. pallida* leaves were collected from Sylhet, Bangladesh whichis a hilly area of the country in July, 2017 and were identified (DACB Accessionno. 42022 & 42023 respectively) by Bangladesh National Herbarium, Dhaka.
Preparation of plant extracts

The crushed plant material was filtered first by using a fresh cotton plug, then by Whatman Filter Papers 125 mm Grade-1 after being soaked in ethanol and cold extracted for seven days. The concentrated extracts were produced by using a rotary evaporator (RE300, Stuart, Japan) set to run at 5 to 6 rpm and 50°C.

Animals

Long Evan rats of either sex, aged 10-11 weeks of average wt. 120-140 g were used for pharmacological screening. The animals were accommodated in colony cages that were maintained at a temperature of 25 ± 2°C, with a relative humidity of 50–55%, and subjected to a 12-hour light/dark cycle. They were provided with rodent feed procured from ICDDR, B, along with water *ad libitum.* All the animals were adapted to the laboratory environments for seven days preceding the investigations. The Southeast University Committee on Ethical Compliance in Research (SEU/Pharm/CECR/104/2021) approved the guidelines that were followed during the entire investigation regarding the treatment of the animals.

In-vitro evaluation
Thrombolytic activity [21, 22]
Preparation of sample and Streptokinase (SK) as standard
To prepare the sample, 10 mg of ethanolic extracts of *H. parasitica* and *C.pallida* were obtained and placed in separate vials. Each vial was subsequently supplemented with 1 mL of distilled water. The thrombolytic activity was determined using a standard of streptokinase (15, 00,000 I.U) obtained from Beacon Pharmaceutical Ltd in Bangladesh. The streptokinase vial was filled with 5 ml of sterilized distilled water followed by thorough mixing. This suspension served as a stock for *in vitro* thrombolysis, of this, 100 µL(equivalent to 30,000 I.U.) was utilized.

Blood Sample Preparation
Blood samples were collected under sterile conditions from selected healthy human volunteers, ensuring they were not using oral contraceptives or anticoagulants. Following blood collection, 1 mL of blood was transferred intopre-weighed, disinfected Eppendorf tubes and incubated at 37°C for 45minutes to allow clot formation.

Thrombolytic activity test:
The serum from the Eppendorf tubes was entirely withdrawn from each Eppendorf tube without disrupting the clot and determined the weight of the clot. The Eppendorf tubes were filled with 100 µl of streptokinase (SK) as a positive control and 100 µl of distilled water as a negative non-thrombolyticcontrol, as well as 100 l of both samples. The discharged liquid was taken from each Eppendorf after incubation at 37 °C for 90 minutes and weighed again to determine the weight of the clot after breakup. Finally, percentage 2 (%) of clot lysis was determined as follows:
% clot lysis = (Weight of the clot after lysis / Weight of clot before lysis) × 100

Membrane stabilizing activity test [23]
Ranasinghe *et al*. described the method to study the membrane stabilizing activity by using Red Blood Cells.
Preparation of phosphate buffer, isotonic and hypotonic solution
Monosodium phosphate (0.90 g) and disodium phosphate (0.2750 g) were used to prepare the sodium phosphate buffer with a pH 7.43. NaCl (4.5045 g) was added to the buffer solution to create a 500 ml isotonic solution with strength of 154 mM, and 1.4625 g of NaCl was added to prepare 500 5ml hypotonic solution with a strength of 50 mM.

Preparation of the sample solution and Erythrocyte suspension

To make the sample solution, 2 mg ethanol extracts of *H. parasitica* and *C.pallida* were dissolved in 5 ml hypotonic buffer solution. Using an anticoagulant called EDTA; human red blood cells were obtained in a test tube at a standard temperature of 25°C and relative humidity of 50-55 percent. The collected blood was washed three times using an isotonic solution (154 mM NaCl) prepared in a 10 mM sodium phosphate buffer at pH 7.4, followed by centrifugation at 3000 rpm for 10 minutes.

Effect on hemolysis induced by hypotonic solution

A volume of 5 ml buffered saline solution (pH 7.4) with a low salt concentration (50 mM NaCl) with the extract or the reference standard, acetyl salicylic acid (0.1 mg/ml) was combined with 0.5 ml of erythrocyte suspension. At room temperature both mixtures were centrifuged at 3000 rpm for 10 minutes, after incubation for 10 minutes. The absorbance (Ab) of the supernatants for the extractives and streptokinase were measured at 540 nm. Using the following formula, the percentage (%) inhibition of hemolysis was determined:

$$\text{Hemolysis inhibition (\%)} = 100 \times \{(Ab_C - Ab_T) / Ab_C\}$$

Where, Ab_C is the absorbance of hypotonic-buffered solution alone (control) and Ab_T is the absorbance of the extractives / reference standard in hypotonic-buffered solution.

Effect on hemolysis induced by heat

5 mL of isotonic-buffered solution (154 mM) having 1.0 mg/mL of both the plant extracts and the streptokinase were centrifuged separately. Each tube received 30 mL of erythrocyte in the form of suspension, which was gently mixed by downturn. In a water bath, one set of test tubes was incubated at 54°C for 20 minutes, while another set of test tubes was kept in an ice bath for 20 minutes, at a temperature of 0-5°C. The test tubes were centrifuged at 1300 rpm for 3 minutes, and absorbance at 540 nm was measured to calculate the percentage inhibition of hemolysis for the test samples/reference standard by the following equation:

Hemolysis inhibition (%) = 100 x [1- (Ab_H - Ab_U / Ab_C - Ab_U)]

Where, Ab_U = test sample unheated, Ab_H = test sample heated and Ab_C = control sample heated

In-vivo evaluation

Animal grouping and dosing

The rats were randomly divided (n = 4) into the following groups and doses:

Group I: received 1 ml/100 mg normal saline (0.9% of NaCl solution)
Group II: treated with loperamide 5 mg/kg for antidiarrheal activity/ aspirin 300mg/kg for antipyretic activity
Group III: administered 100 mg/kg extract *H. parasitica*
Group IV: administered 150 mg/kg extract *H. parasitica*
Group V: administered 200 mg/kg extract *H. parasitica*
Group VI: administered 100 mg/kg extract *C. pallida*
Group VII: administered 150 mg/kg extract *C. pallida*
Group VIII: administered 200 mg/kg extract *C. pallida*

Antidiarrheal activity

The procedure given by Bulbul *et al.*[24] was used to conduct this investigation. 32 rats were divided into five groups of four animals each after fasting for 18 hours. A long needle with a ball shaped end was used to administer saline water (10 ml/kg) to group I, loperamide (5 mg/kg) to group II, and test extracts to groups III, IV, V, VI, VII, and VIII at zero hour. After an hour, each rat in the study received an oral dose of 0.5 cc (mL) of castor oil. The animals were maintained in separate cages with faeces collected in a transparent plastic beneath the cage. For a total of four hours, the intensity of diarrhea was measured every hour. The mean total count of faeces (both the dry and wet diarrheal droppings) in the negative control group was compared with the treated group to determine % inhibition of defecation by using the following equation:

% inhibition of defecation = (NfC − NfT)/ NfC × 100

Nf_C = Total count of faeces in the negative control, Nf_T = Total count of faeces in treated group

The total number of wet faeces for the negative control group was compared with that of the treated group to calculate % of inhibition of diarrhea using the following formula:

% inhibition of diarrhea = (Nwf_C − Nwf_T)/ Nwf_C × 100

Nwf_C = Total count of wet faeces in the negative control, Nwf_T =Total count of wet faeces in treated group

Antipyretic activity

Brewer's yeast-induced pyrexia was used to test the plant extract's antipyretic efficacy, as described by Rajasekaran *et al.*, 2010.[25] Each group of four rats received a different treatment, such as Group-I, which acted as the control and got saline. Aspirin was given to Group II to compare potencies. The crude extract was given orally to Groups III, IV, V, VI, VII, and VIII. Tween-80 and DMSO were used to make suspensions of conventional Aspirin at a dose of 300 mg/kg body weight and extracts at doses of 100, 150, and 200 mg/kg body weight in normal saline. Using normal saline, a 20% aqueous suspension of brewer yeast was initially produced, as per the methodology. The yeast solution was given to all rats at zero hour after a digital rectal thermometer was used to check their body temperature. After 18 hours, the temperature of the rats was examined, and those with an elevated temperature (0.5ºC) were chosen for the antipyretic test. Later, feeding needles were used to deliver normal saline to Group I, aspirin to Group II, and extracts to Groups III, IV, V, VI, VII, and VIII. Finally, from19 to 23 hours after yeast induction, the temperature was recorded at hourly intervals.

Statistical analysis

The data are presented as Mean ± SD. A one-way ANOVA (analysis of variance) conducted by using SPSS version 10.0 was used for the statistical analysis and group comparison, and the LSD (Least Significance Difference) test was then used. A *p*-value below 0.05 was considered indicative of a statistically significant difference between unexposed and exposed groups (with or without treatment).

Results and Discussion

Results of Thrombolytic activity

H. parasitica (HP) showed 75.93% and *C. pallida* (CP) showed 73.64% inhibition of clot lysis while the inhibition of clot lysis for Streptokinase was 85.07%. From this experiment, it can be concluded that *H. parasitica* and *C.pallida* showed very good thrombolytic activity compared to the streptokinase (Figure, 1). The end effect of the blood coagulation stage in hemostasis is athrombus, also known as a blood clot. Thrombus is the hypercritical reason for vascular diseases like hypoxia, hypertension, myocardial infarction, cerebrovascular diseases, etc. Myocardial infarction and cerebral venous sinust hrombosis (CVST) are considered as 1the chief reasons for mortality worldwide.[26] Many research in the 20th century discovered several synthetic antithrombotic molecules comprises warfarin, aspirin, clopidogrel, and heparin which are widely consumed medications to prevent cerebrovascular and cardiovascular diseases. Thrombolytic medications, for example, tissue plasminogen activator (t-PA), streptokinase, urokinase, and others are vital medicines in the treatment of CVST patients.[27] Synthetic thrombolytic drugs have adverse effects such as allergic reactions, anaphylactic shock, hypotension, angioedema, reperfusion arrhythmias, reduced fibrin specificity,and the most frequently the bleeding tendency.[28, 29] A review on thrombolytic investigation of many herbal plants found that they can be used to treat cerebrovascular and cardiovascular diseases efficiently without producing any severe adverse effects.[30] In this study, *H. parasitica* and *C. pallida* were found to have thrombolytic activity and as they are from natural sources, it can be said that they may be the alternate therapy for the thrombus related diseases and free of severe adverse effects.

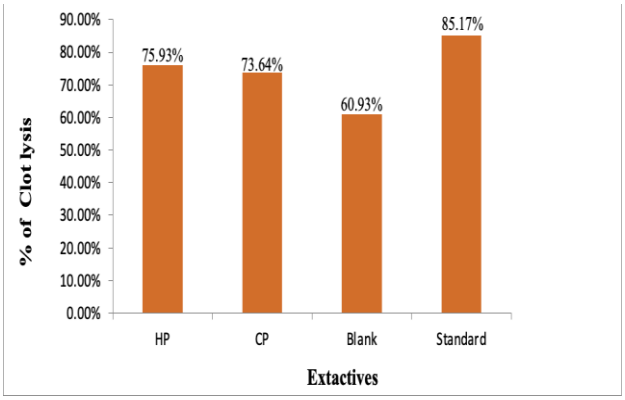

Figure 1: Thrombolytic Activity (% of clot lysis) of the extractives of *H. parasitica* & *C. pallida*

Results of Membrane Stabilizing Activity

Figure 2 shows that, in hypotonic solution-induced hemolysis *H. parasitica* showed 64.35% and *C. pallida* showed 68.23% inhibition of hemolysis of RBC compared to acetyl salicylic acid that exhibited 79.68% inhibition of hemolysis. Both the plant extracts were effective in the membrane stabilizing activity and they prevented the lysis of erythrocytes induced by heat. In heat-induced hemolysis *H. parasitica* showed 62.89% and *C. pallida* showed 68.14% inhibition of hemolysis of RBC while the acetyl salicylic acid exhibited 74.49% inhibition of hemolysis. *H. parasitica* and *C. pallida* demonstrated excellent membrane stabilizing action in this investigation. By blocking the release of lysosomal contents that promote additional tissue inflammation, lysosomal membrane stabilization is vital in minimizing the inflammatory process. The human red blood cells (HRBC) membrane is similar to the lysosomal membrane. The medications or plant extracts having ability 4tostabilize the membrane of HRBC through the inhibition of hypotonic-solution5 and heat-induced membrane lysis may be explained to have anti-inflammatory activity. Inflammation is the natural immunological response of the body against infections, injuries, and toxins to heal. Inflammatory cells create a complex mixture of cytokines and physiologically active

arachidonate metabolites in chronic inflammation, which has been linked to aging,

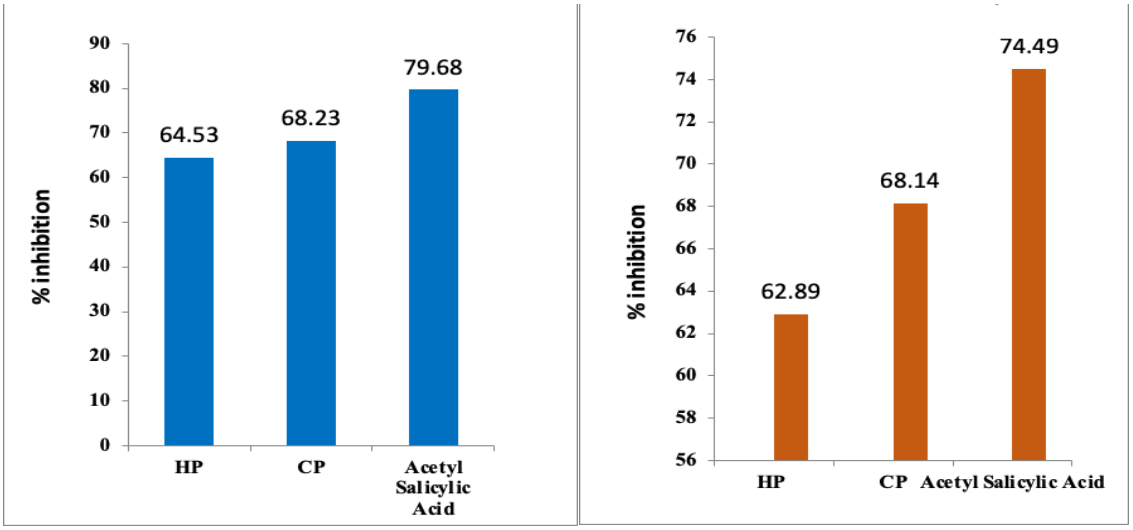

Figure 2: Effects of the extracts of *H. parasitica* (HP) and *C. pallida* (CP) on hypnotic solution and heat induced hemolysis

adipogenesis, cancer, cardiovascular problems, diabetes, lung ailments, and some other diseased conditions.[31, 32] As a result of their high membrane stabilizing activity, these plants may have anti-inflammatory properties. By stabilizing the membrane, they may prevent the leakage of inflammatory chemicals. Furthermore, these inflammatory cells can produce reactive oxygen species, which can harm cellular biomolecules.[33] *C. pallida* and *H.parasitica* both were found to have very good membrane stabilizing action, and proved to have anti-inflammatory properties. The presence of flavonoids[16,18] in *C. pallida* may be the reason for it's membrane stabilization as well as anti-inflammatory effect. For both plants thrombolytic and membrane stabilizing activity were investigated for the first time in this study.

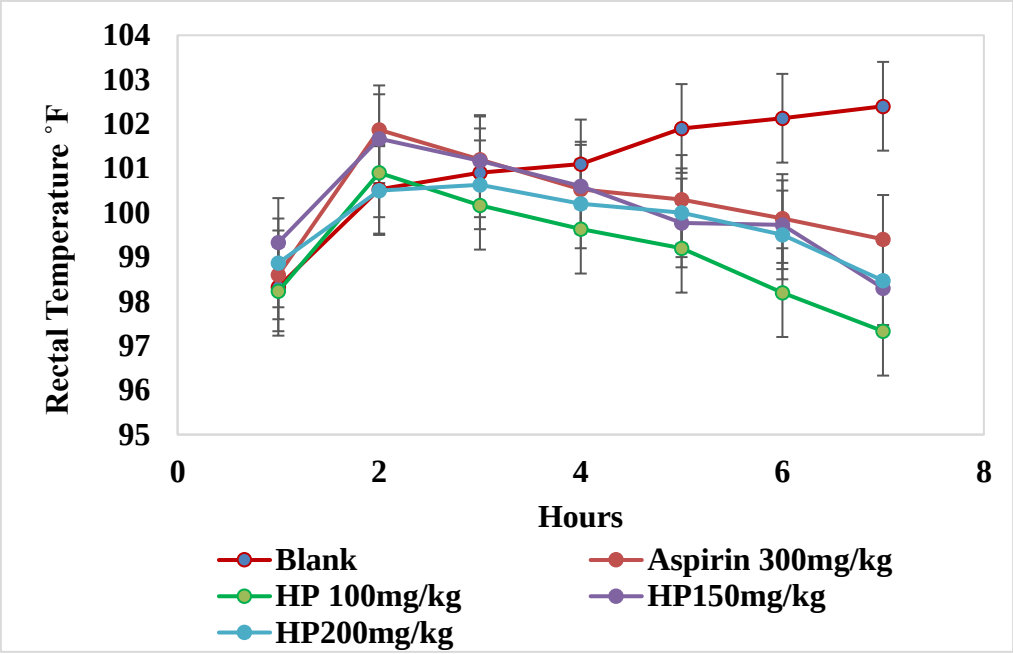

Figure 3: Antipyretic effects of ethanol extracts of *H. parasitica* in pyrexia induced rats

Result of anti-diarrheal activity induced by castor oil

The result of the antidiarrheal activity of *H. parasitica* and *C. pallida* leaves are shown in Table-18. In the normal group, the number of wet (diarrheal) stools was increased after induction of diarrhea with castor oil. Loperamide was used as standard control at a dose of 5 mg/kg and decreased the number of wet (diarrheal) stool and the number of total stool significantly ($P<0.05$) during the treatment period (Table 1). In the treatment group, the number of wet stool and the number of total stool was significantly ($P<0.05$) reduced compared to normal control for both the ethanolic extracts of *H. parasitica* and *C.pallida* leaves at different doses (100, 150, and 200 mg/kg body weight). Diarrhea is a leading cause of mortality in developing countries, predominantly impacting children and new-borns. There are a large number of herbal medications that are reported to be beneficial in treating diarrhea all over the world. In the current investigation,[34] both plant extractives inhibited the frequency of wet faeces in an animal model in a dose-dependent manner,revealed that both the plants may have anti-diarrheal potential.

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

Table 1: Effect of ethanol extract of leaves of *H. parasitica* (HP) and *C. pallida* (CP) on castor oil induced diarrhea in rat

Treatment	No. of wet stools	No. of hard stool	Total no of stool	% inhibition of diarrhea
Normal	5.75 ± 0.47	2.00 ± 0.40	7.75 ± 0.47	
Loperamide 5mg/kg	2.25 ± 0.25*	4.25 ± 0.64*	6.75 ± 0.62	60.87
HP 100 mg/Kg	2.00 ± 0.40*	3.25 ± 0.62	5.25 ± 0.62*	65.22
HP 150 mg/Kg	1.50 ± 0.28*	3.00 ± 0.91	4.50 ± 0.86*	73.91
HP200 mg/Kg	0.75 ± 0.47*	2.75 ± 0.25	3.50 ± 0.64*	86.96
CP 100 mg/Kg	1.75 ± 0.25*	3.00 ± 0.40	4.75 ± 0.25*	69.57
CP 150 mg/Kg	1.50 ± 0.28*	2.25 ± 0.47	3.75 ± 0.52*	73.91
CP 200 mg/Kg	0.50 ± 0.28*	1.75 ± .47	2.25 ± 0.47*	91.30

Values are mean ± S.E.M (n = 4), * for $p < 0.05$ compared to the negative control

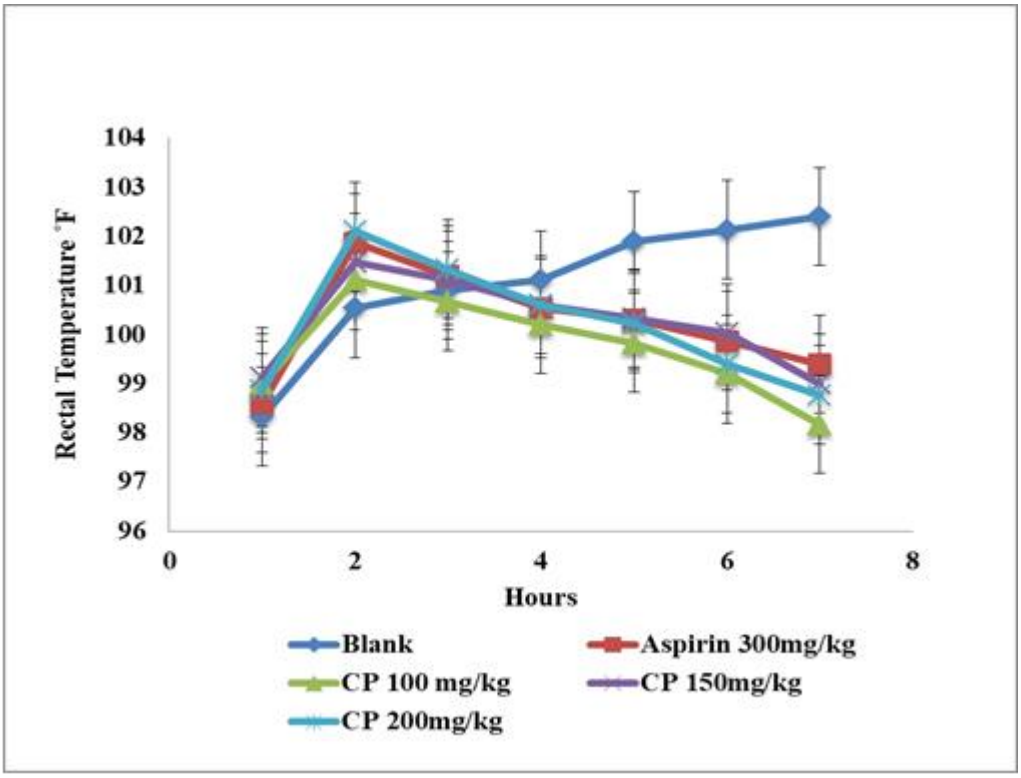

Figure 4: Antipyretic effects of ethanol extracts of *C. pallida* in pyrexia induced rats

Result of Brewer Yeast Induced Pyrexia Test

Figures 3 and 4 indicate the antipyretic action of the leaves of *H. parasitica* and *C. pallida*. After pyrexia was induced with Brewer's yeast, the temperature inthe control group increased from 98.38 of 12to 102.53 °F. The temperature was lowered from 98.62 of 13 to 99.40 °F four hours after administration with aspirin at a dose of 300 mg/kg in this trial. At a dose of 100 mg/kg body weight, ethanolic extracts of *H. parasitica* leaves reduced temperature from 98.12 to 97.32 °F,150 mg/kg reduced temperature from 99.22 to 98.3 °F, and 200 mg/kg reduced temperature from 99.12 to 98.8 °F at the 21st, 22nd, and 23rd hour of treatment period considerably compared to the initial. When *C. pallida* was given at a dose of 100 mg/kg, the

temperature was reduced significantly from 99.00 to 98.35 °Fat the 23rd hour, from 99.00 to 98.97 °F at a dose of 150 mg/kg, and from 98.90to 98.85 °F at a dose of 200 mg/kg at the four-hour treatment period, compared to the initial temperature. This study found that *H. parasitica* and *C. pallida* have anti-pyretic action in an animal model. The fact that the ethanolic extracts of *H.parasitica* and *C. pallida* lowered the high temperature in the yeast model backs with the assertion that these two plants have long been used to cure fever. Acetyl salicylic acid was utilized as the reference antipyretic medication in the brewer yeast generated models, and it works by significantly reducing prostaglandin (E-type) protection in the hypothalamus.[35] The extract's antipyretic effect may be attributed to its inhibition of prostaglandin synthesis,which results in the control of high plasma levels. *H. parasitica* was previously reported for its anti-diarrheal and antipyretic activities [12] and this study supported the previous findings, while for *C. pallida*, anti-diarrheal, and antipyretic activities were reported for the first time.

Conclusion

According to the findings, ethanolic extracts of *H. parasitica* and *C. pallida* have thrombolytic, membrane-stabilizing, antipyretic, and antidiarrheal activities.

Conflict of interest

The authors declare that there is 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.

Acknowledgements

Authors would like to acknowledge the institution of research and training,Southeast University for providing partial funding of this research (SEU/IRT/2017-80).

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