



(RESEARCH ARTICLE)



Antioxidant, anti-inflammatory and anti-hyperglycemic activity of aqueous and methanolic extract of *Houttuynia cordata*: an *in vitro* and *in vivo* study

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Abstract

Available synthetic antioxidants such as butylated hydroxyl anisole, butylated hydroxyl toluene, propyl gallate, and ascorbic acid exhibit several side effects. To curb these side effects, more effective, less toxic, and cost-effective drugs are required. Therefore, this study aims to screen and evaluate the antioxidant as well as the anti-inflammatory and antidiabetic potential of *Houttuynia cordata* collected from Mairang village, West Khasi Hills, Meghalaya, India using several standard methods. The aqueous and methanolic extracts of *H. cordata* were evaluated by screening their ability to scavenge 1,1-diphenyl-2-dipicrylhydrozyl, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), hydrogen peroxide, and nitric oxide. Total phenol and flavonoid content was measured by Folin-Ciocalteu and by AlCl₃ colorimetric method respectively. The anti-inflammatory activity of the plant was determined using the protein denaturation method. Methanolic and aqueous extracts of *Houttuynia cordata* exhibit varying free radical scavenging and anti-inflammatory activity. Among the extracts used in the study, the methanolic extract of *Houttuynia cordata* elicited a higher activity than the aqueous extract. *Houttuynia cordata* also elicited a marked reduction in blood glucose level of normal and alloxan-induced diabetic mice. Flavonoids, which have been reported to possess anti-inflammatory, enzyme inhibition, antimicrobial, anticancer, antiallergy, and antioxidant properties may contribute towards the free radical scavenging and anti-inflammatory effect of *Houttuynia cordata*.

Keywords: Anti-hyperglycemic; Anti-inflammatory; Antioxidant; *Houttuynia cordata*

1. Introduction

Oxidative stress can lead to chronic inflammation if there is an imbalance between the production of reactive oxygen species (ROS) and their elimination [1]. It activates transcription factors which in turn alters the expression of certain genes involved in the inflammatory pathway [1]. Inflammation is a factor that is closely involved in the pathogenesis of type 2 diabetes [2]. Therefore, novel anti-inflammatory treatments for diabetes need to be tested. Natural compounds in plants like polyphenols can interact with ROS/RNS and modulate their inflammatory response [3]. Phenols and flavonoids found in all parts of plants such as leaves, fruits, seeds, roots and bark are potent free radical scavengers [4] that protect the body from various diseases by terminating the action of free radicals [5].

Oxidative stress is an important underpinning phenomenon that assists with the progression towards more severe and often fatal complications [6]. Evidence from numerous studies suggests that ROS particularly, Mitochondrial ROS play an important role in diabetes and its associated complications [7]. High blood glucose leads to production of free radicals via autoxidation of glucose and an increased flux through the polyol and hexosamine pathway [8]. Autoxidation of glucose accelerates formation of advanced glycated end products (AGEs). Cross linking AGE protein with other molecules results in cell and tissue abnormalities [9].

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Natural resources have provided leads to drug discovery [10] and have drawn a lot of attention in recent years. They are the richest source of inspiration for the identification of novel scaffold structures that can serve as the basis of rational drug design [11]. In most developing countries, conventional drugs are used by 70-95 % of the population for primary health care, and 85% of these people use plants or their extracts as the active ingredient. Several epidemiological studies have suggested that plants rich in antioxidants play a role in health and against several diseases. Their consumption reduces the risks of heart diseases, stroke, hypertension, cancer, etc [12-13]. Natural antioxidants inhibit ROS generation by scavenging free radicals and altering redox potential [14]. Polyphenols such as phenolic acids, and flavonoids are potent antioxidants [15]. In view of the widespread interest on medicinal plants, the present study on *Houttuynia cordata* provides information of its phytochemical and pharmacological activity.

2. Material and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid (Trolox), 2, 2-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS), ascorbic acid, and rutin were purchased from Sigma Chemicals Co. (St. Louis, USA). All other chemicals used were of analytical grade purchased from Merck, Himedia, and SRL, India.

2.2. Plant collection and extraction

Leaves of *Houttuynia cordata* (Voucher no.NEHU-11922) were collected from Mairang village, West Khasi Hills, Meghalaya. The plant material was submitted and identified by herbarium curator Dr. P.B. Gurung, Department of Botany, NEHU, Shillong. Fresh leaves were washed, dried, and powdered using an electric blender. The dried powder was then extracted in 80% methanol in the ratio of 1:10 (plant: solvent) [16]. The mixture was filtered using a muslin cloth followed by whattmann no.1. The filtrate was concentrated in a rotary evaporator (Yamato RE800) and then lyophilized (ScanvacCoolsafe) to obtain the crude powder (gm). The crude powder was weighed to calculate the percentage yield and stored at 4° for further usage.

2.3. Experimental animals

Swiss albino mice (Balb/C strains) weighing 25-30 g, procured from Pasteur Institute, Shillong, Meghalaya were used for the study. Mice were housed in a room kept under controlled conditions maintained at a temperature of 22° C on a 12 h light/dark cycle. The mice were fed with balanced mice feed obtained from Pranav Agro Ltd., New Delhi, India. All The experiments were conducted after the approval by the Institutional Ethics Committee (IEC) (Dated: 01.10.2018) of North-Eastern Hill University, Shillong, Meghalaya, India.

2.4. Preparation of diabetic mice

Healthy mice weighing 25-30 grams were administered with alloxan monohydrate prepared in 0.15M acetate buffer, pH 4.5 intravenously at a dose of 80 mg/kg body weight. Prior to induction mice were starved overnight but given water *ad libitum*. After 48h, mice with more than 3-4 fold increase in blood glucose (measured using glucoStix; SDCheck) were considered diabetic and used for the study.

Mice were divided into 4 groups:

NC: Normal control

DC: Diabetic control

MHC: Diabetic mice treated with methanolic extract of *H. cordata*

AHC: Diabetic mice treated with aqueous extract of *H. cordata*

2.5. Toxicity Tests

Organization for Economic Cooperation and Development (OECD) guidelines [17] were followed in order to determine the toxicity of the plant extracts (if any). Mice provided with ad libitum were starved for 4h. For the limit test, five mice were administered with a limit dose of 2000 mg/kg b.w. plant extract. If the animal died, the main test was conducted

to determine the lethal dose at which 50% of the mice died (LD₅₀). In addition, the mice were observed for any signs of distress, convulsion, coma or death.

2.6. Total Phenolic content

Total phenolic content was determined according to Singleton [18]. The plant extract was reacted with a Folin-Ciocalteu reagent and incubated with 7.5% sodium carbonate in the dark at room temperature for 2h. Following incubation, absorbance was measured at λ 740 nm and the results were expressed as mg gallic acid equivalent (GAE)/ gm dry weight of the extract.

2.7. Total Flavonoid content

Total flavonoid content was determined according to Kosalec [19]. Plant extract or standard was reacted with 95% ethanol, 10% Aluminium chloride, and 1M potassium acetate and incubated for 30 min at room temperature. Following incubation, absorbance was measured at λ 415 nm and the results were expressed as mg Quercetin equivalent (QE)/ gm dry weight of the extract.

2.8. Antioxidant assay

2.8.1. DPPH assay

The DPPH radical scavenging potential of the plant extracts was determined according to Brand-Williams [20]. Varying concentration of plant extract or standard was reacted with 0.004% DPPH and allowed to stand for 30 min at room temperature. Following incubation, absorbance was measured at λ 517 nm against a blank solution. Ascorbic acid was used as standard. DPPH radical scavenging activity was calculated as:

$$\text{Scavenging Percentage} = \frac{\text{Abs (control)} - \text{Abs (Sample)}}{\text{Abs (control)}} \times 100$$

2.8.2. Hydrogen peroxide (H₂O₂) assay

The hydrogen peroxide scavenging potential of the plant extracts was determined according to Jayaprakash [21]. 20 mM Hydrogen peroxide solution was reacted with different concentrations of the plant extracts or standard and incubated for 10 min. Following incubation, the absorbance was measured at λ 230 nm against a blank solution. The scavenging percentage was calculated as above.

2.8.3. Nitrogen oxide (N₂O) assay

Nitric oxide scavenging potential of the plant extracts were examined according to Green [22] 10mM sodium nitroprusside, phosphate buffer saline, and extract or standard solution was incubated at 25°C for 150 min. From the reaction mixture, 0.5ml was taken followed by the addition of 0.5ml 0.33% sulphanic acid reagent. This was vortexed and allowed to stand for 5 min. Following the diazotization reaction, 0.1% NEDD was added and allowed to stand for 30 min in diffused light. The absorbance was then measured at 546nm against the corresponding blank solution. The scavenging percentage was calculated as above.

2.8.4. Total antioxidant assay

The total antioxidant capacity of the plant extracts was measured determined according to Kannan [23]. Varying concentration of plant extract or standard was reacted with reagent solution containing 28mM sodium phosphate, 4mM ammonium molybdate, and 0.6M sulfuric acid and incubated for 90 min at 95°C. Following incubation, absorbance was read at 695 nm against a blank. The activity was expressed in mg ascorbic acid equivalent (AAE)/ gm dry weight of the extract. Rutin was used as a reference standard.

2.8.5. Trolox equivalent antioxidant (TEAC) assay

The TEAC of the plant extracts was measured using the method described by Rubio [24]. Varying concentrations of trolox or plant extract were reacted with ABTS radical cation and incubated at room temperature for 4 minutes. Following incubation, absorbance was read at 734nm against a blank solution. Inhibition of ABTS radical scavenging activity was calculated as above. The scavenging activity was expressed in mg trolox equivalent/ gm dry weight of the extract.

2.9. Anti-inflammatory assay

The anti-inflammatory potential of the plant extracts was determined according to Dey [25]. Varying concentrations of plant extract or standard were reacted with egg albumin and phosphate-buffered saline (pH 6.4). The mixture was incubated at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 15 min and then heated at 70°C for 5 min. The absorbance was then measured at 660 nm against the corresponding blank. Aspirin was used as a reference standard. The percentage inhibition of protein denaturation was calculated as above.

2.10. Normoglycemic and antihyperglycemic study

Normal and alloxan-induced diabetic mice were starved overnight prior to the experiment. Crude plant extract was administered via the intraperitoneal route to the test groups. The control group received only distilled water. Food was withheld during the experiment period [26].

2.11. Statistical analysis

All analyses were carried out in triplicates. Data were presented as mean \pm SD. To evaluate significant relationships between experimental parameters by correlation and regression analysis, *t*-tests (p-value <0.001) was used.

3. Results

3.1. Yield Percentage

From 10 gm crude powdered plant, the percentage yield of methanolic and aqueous extract of *H. cordata* was 13.80% and 5.6% respectively.

3.2. Toxicity

The LD₅₀ and toxic dose of the plant extract was found to be 750 mg/kg b.w. and 950 mg/kg b.w. respectively.

3.3. Total Phenolic content

The total phenol content of methanolic and aqueous extract of *H. cordata* was found to be 256.79 ± 0.45 mg GAE/ gm of dried extract and 91.25 ± 0.08 mg GAE/ gm of dried extract respectively.

3.4. Total Flavonoid content

The flavonoid content of methanolic and aqueous extract of *H. cordata* was found to be 127.27 ± 0.33 mg QE/ gm of dried extract and 62.659 ± 0.20 mg QE/ gm of dried extract respectively.

3.5. Antioxidant assay

Methanolic and aqueous extract of *H. cordata* exhibited varying antioxidant scavenging activity. Methanolic extract of *H. cordata* was found to be more active than its aqueous counterpart in scavenging free radicals. The IC₅₀ value for DPPH, NO₂, and H₂O₂ was 47.94 ± 0.60 , 28.974 ± 0.27 , and 113 ± 0.78 respectively (Table 1). The TAC and TEAC activity was 296.23 ± 0.67 mg ascorbic acid equivalent/gm dry weight and 383.95 ± 2.60 mg ascorbic acid equivalent/gm dry weight respectively (Table 2).

Table 1 *In vitro* DPPH, H₂O₂ and NO₂ Scavenging assay activity of *H. cordata* and ascorbic acid. Values are reported as M \pm SEM. M: Mean, SEM: Standard error of mean.

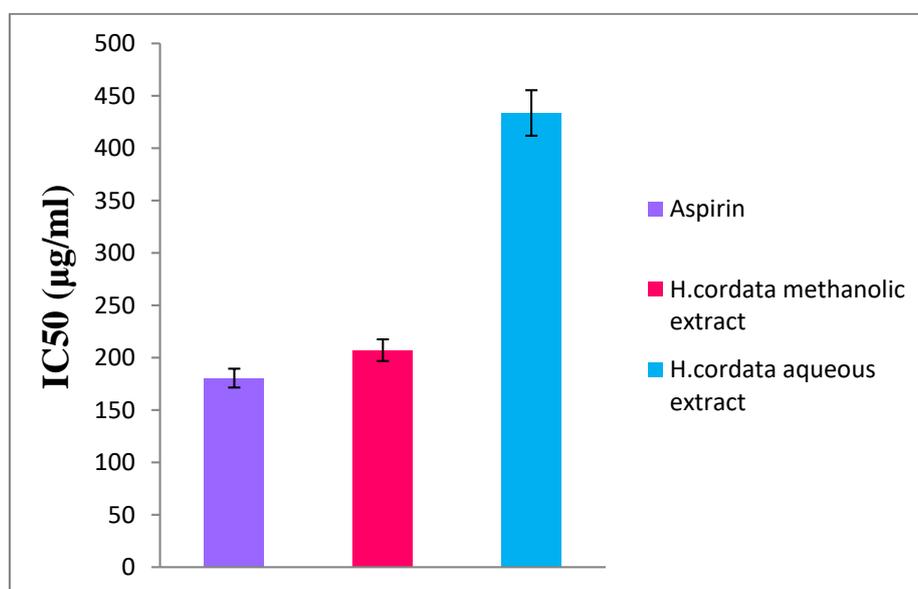
Plant extract / Reference Standard	DPPH Scavenging assay IC ₅₀ ($\mu\text{g}/\text{ml}$) M \pm SEM	H ₂ O ₂ Scavenging assay IC ₅₀ ($\mu\text{g}/\text{ml}$) M \pm SEM	NO ₂ Scavenging assay IC ₅₀ ($\mu\text{g}/\text{ml}$) M \pm SEM
<i>H. cordata</i> methanolic extract	47.94 ± 0.60	113.01 ± 0.78	28.974 ± 0.2733
<i>H. cordata</i> aqueous extract	109.99 ± 0.50	236.36 ± 0.65	33.099 ± 0.50
Ascorbic acid	6.7 ± 0.01	27.75 ± 0.03	6.86 ± 0.13

Table 2 *In vitro* total antioxidant capacity and trolox equivalent antioxidant activity of *H. cordata* and rutin. Values are reported as M ± SEM. M: Mean, SEM: Standard error of mean.

Plant extract / Reference Standard	Total Antioxidant Capacity (mg ascorbic acid equivalent/gm dry weight) M±SEM	Trolox Equivalent Antioxidant Capacity (mg ascorbic acid equivalent/gm dry weight) M±SEM
<i>H. cordata</i> methanolic extract	296.23 ± 0.67	383.95 ± 2.60
<i>H. cordata</i> aqueous extract	104.94 ± 0.59	237.65 ± 3.44
Rutin	362.93 ± 0.89	156.44 ± 0.60

3.6. Anti-inflammatory assay

In the anti-inflammatory assay, methanolic extract ($207.16 \pm 1.43 \mu\text{g/ml}$) was found to be more active than the aqueous extract of *H.cordata* ($433.66 \pm 2.57 \mu\text{g/ml}$)(Figure 1). The IC₅₀ value of the methanolic extract was comparable to aspirin ($180.52 \pm 0.57 \mu\text{g/ml}$).

**Figure 1** *In vitro* anti-inflammatory activity of *H.cordata* and aspirin. Values are reported as M ± SEM. M: Mean, SEM: Standard error of mean.

3.7. Normoglycemic study

3.7.1. Aqueous extract of *Houttuynia cordata*

Normal mice treated with varying doses (150-450mg/kg b.w) of aqueous extract of *H.cordata* showed a reduction at all time intervals studied (Table 3 and figure 2). The hypoglycemic effect was observed for all doses used. However, the maximum reduction was seen at a dose of 450 mg/kg b.w. decreasing the glucose level by 46% ($p < 0.001$), 45% ($p < 0.001$), 56% ($p < 0.001$) and 11% at 2, 4, 6 and 24 h respectively from that of the control.

Table 3 Blood glucose levels and % increase/ decrease brought about by aqueous extract of *H.cordata* on normal mice at different time intervals. Values are reported as M ± SEM. ** represents level of significance at **p<0.01, and *** represents level of significance at ***p<0.001 compared against normal control group. NS: non-significant, M: Mean, SEM: Standard error of mean, NC: normal control.

Groups	2h	4h	6h	24h
NC	113±3.00	104.5±0.25	105.5±2.25	118±2.40
<i>H. cordata</i> aqueous extract (150 mg/kg b.w)	72.3±5.37	67±3.20	62±3.05	115.5±1.50
<i>H. cordata</i> aqueous extract (250 mg/kg b.w)	61.3±2.14	58.3±5.46	56±2.30	110.5±0.75
<i>H. cordata</i> aqueous extract (450 mg/kg b.w)	60±3.00	57±5.73	46±2.13	105±2.03

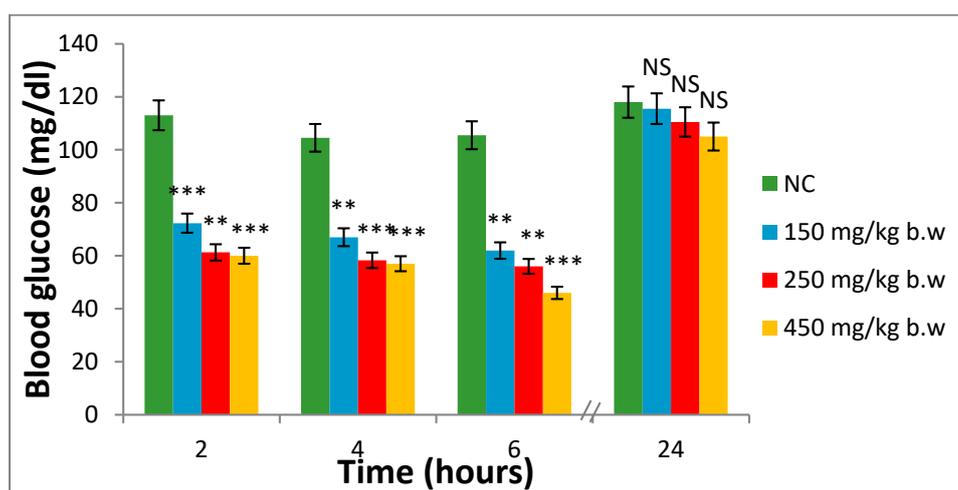


Figure 2 The effect of varying doses of aqueous extract of *H. cordata* on normal mice at different time intervals. Values are reported as M ± SEM. ** represents the level of significance at **p<0.01, and *** represents the level of significance at ***p<0.001 compared against the normal control group. M: Mean, SEM: Standard error of mean, NC: normal control.

3.7.2. Methanolic extract of *Houttuynia cordata*

Intraperitoneal administration of varying doses (150-450mg/kg b.w) of the methanolic extract to normal mice elicited a hypoglycemic effect (Table 4 and figure 3).

Table 4 Blood glucose levels and % increase/ decrease brought about by methanolic extract of *H.cordata* on normal mice at different time intervals. Values are reported as M ± SEM. p: * represents level of significance at *p<0.05, **p<0.01 and ***p<0.001 compared against normal control group. M: Mean, SEM: Standard error of mean, NC: normal control.

Groups	2h	4h	6h	24h
NC	113±3.00	104.5±0.25	105.5±2.25	118±2.40
<i>H. cordata</i> methanolic extract (150 mg/kg b.w)	77.33±5.17	72±5.03	67.33±3.28	77.66±1.68
<i>H. cordata</i> methanolic extract (250 mg/kg b.w)	73.66±1.33	66±1.52	51.667±2.33	74.66±1.76
<i>H. cordata</i> methanolic extract (450 mg/kg b.w)	94±7.00	68±2.00	57±3.00	42±5.00

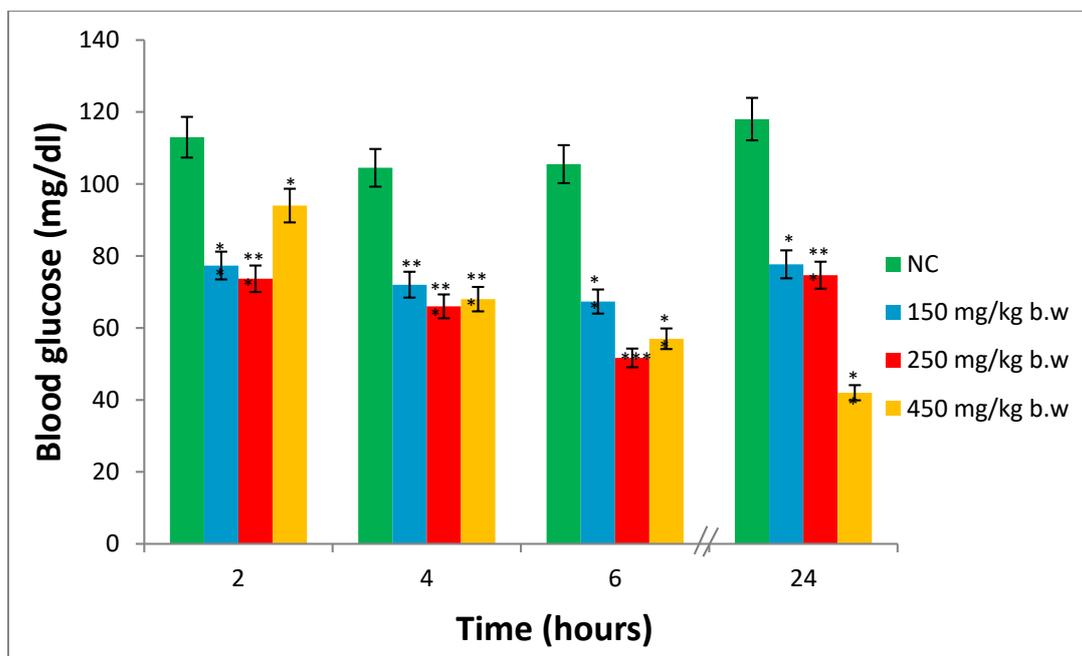


Figure 3 The effect of varying doses of methanolic extract of *H.cordata* on normal mice at different time intervals. Values are reported as $M \pm SEM$. ** represents the level of significance at $**p < 0.01$, and *** represents the level of significance at $***p < 0.001$ compared against the normal control group. NS: nonsignificant, M: Mean, SEM: Standard error of mean, NC: normal control.

For the doses studied, a more pronounced blood glucose was seen dose of 250 mg/kg b.w. decreasing the glucose level by 34% ($p < 0.001$), 36% ($p < 0.001$), 54% ($p < 0.001$) and 36% ($p < 0.001$) at 2, 4, 6 and 24 h respectively from that of the control.

3.8. Antihyperglycemic study

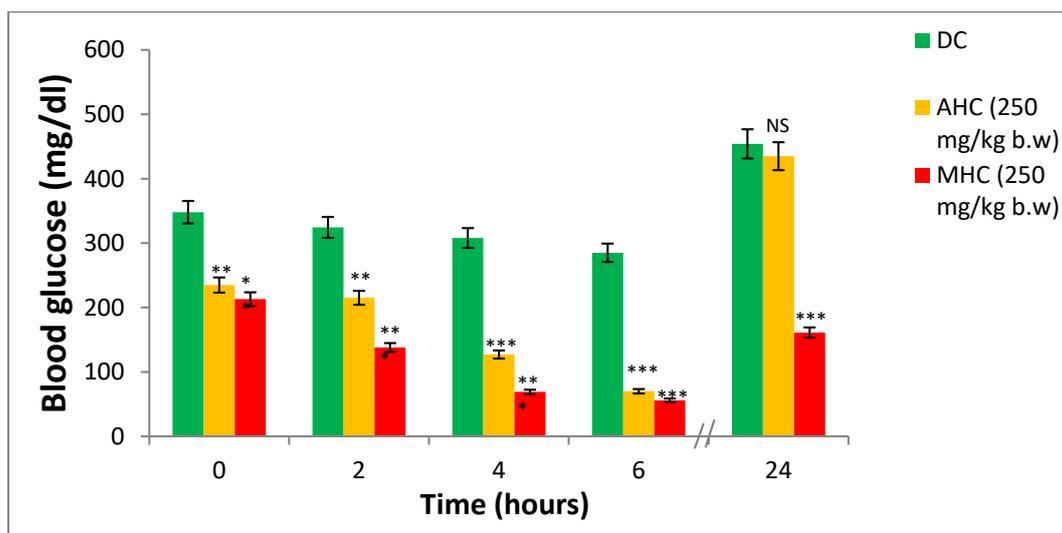


Figure 4 The effect of varying doses of AHC and MHC on alloxan-induced diabetic mice at different time intervals. Values are reported as $M \pm SEM$. ** represents the level of significance at $**p < 0.01$, and *** represents the level of significance at $***p < 0.001$ compared against the diabetic control group. NS: nonsignificant, M: Mean, SEM: Standard error of mean, DC: diabetic control, AHC: diabetic mice treated with aqueous extract of *H.cordata*, MHC: diabetic mice treated with aqueous extract of *H.cordata*.

Based on the normoglycemic study, 450 mg/kg b.w of AHC was potent in bringing down the blood sugar level, however, it was also observed that at this dose, the mice exhibited a hypoglycemic condition. Hence, 250 mg/kg b.w. of aqueous

extract of *H.cordata* was chosen for the antihyperglycemic study. AHC at a dose of 250 mg/kg b.w as well as MHC at a dose of 250 mg/kg b.w elicited a reduction in blood glucose level of alloxan-induced diabetic mice (table 5 and figure 4). The anti-hyperglycemic activity of MHC was more pronounced with a reduction of 57% ($p<0.001$), 77% ($p<0.001$), 80% ($p<0.001$) and 64% ($p<0.001$) at 2, 4, 6 and 24 h respectively.

Table 5 Blood glucose levels and % increase/ decrease brought about by AHC and MHC on alloxan-induced diabetic mice at different time intervals. Values are reported as $M \pm SEM$. ** represents the level of significance at $**p<0.01$, and *** represents the level of significance at $***p<0.001$ compared against the diabetic control group. NS: nonsignificant M: Mean, SEM: Standard error of mean, DC: diabetic control, AHC: diabetic mice treated with aqueous extract of *H.cordata*, MHC: diabetic mice treated with aqueous extract of *H.cordata*.

Groups	0.5h	2h	4h	6h	24h
DC	348±24	324.5±11.50	308±8.00	285±15.00	454±15.00
AHC (250 mg/kg b.w)	235±3.60	215±2.64	127±2.30	70±0.88	435±4.04
MHC (250 mg/kg b.w)	213± 0.57	138±8.08	69±6.64	56±6.08	161±9.45

4. Discussion

In this study, aqueous & methanolic extracts of *H.cordata* were examined and evaluated for their antioxidant, anti-inflammatory, and antihyperglycemic potentials. MHC exhibited the highest antioxidant, anti-inflammatory, and antihyperglycemic activity, whilst aqueous extract was significantly less effective. MHC was also found to possess the highest TPC and TFC. It is known that the majority of the antioxidant and anti-inflammatory activities of plants are contributed mainly by flavonoids and polyphenols [27]. According to previous researches, phenolic compounds with ortho- and para- dihydroxylation or a hydroxy and a methoxy group or both have stronger antioxidant activity than simple phenolics [28]. The presence of ketone groups as well as a conjugated double bond in the whole molecule might play different polarities in the structure of the antioxidants and can be attributed to their antioxidant activity [29]. The other factor is related to the sensitivity of Folin-Ciocalteu reagent to a broad range of phenolic compounds whereas the DPPH free radicals show different sensitivity to various antioxidants. The Folin-Ciocalteu reagent reacts to both free phenolics and bound phenolics in extracts and other samples, but the DPPH assay determines free antioxidants and phenolics [13]. Hence, the variations in the TPC content and the antioxidative potential. Greater TEAC and TAC values of MHC can be indicative of their phenolic and flavonoid content.

For classifying a plant as one with an antidiabetic potential it should be able to lower blood glucose elevation. In the present study, both aqueous & methanolic extracts of *H.cordata* had a significant blood-glucose-lowering effect. The effect of the extracts varied in their effects in a time and dose-dependent manner, however, the methanolic extract was more potent. The extract had a maximum reduction of 64% ($p<0.01$). According to Patel, plants belonging to the family Leguminosae, Lamiaceae, Liliaceae, Curcubitaceae, Asteraceae, Moraceae, Rosaceae, Euphorbiaceae, and Araliaceae had a potent hypoglycemic effect [30]. Incidentally, *H.cordata* belongs to the family Asteraceae which thereby supports its potent glucose-lowering potential in normal mice. According to studies flavonoids are known to exert hypoglycemic effects which are useful in the treatment of diabetes [31]. A prolonged antihyperglycemic effect was also observed for the MHC (Table 5 & Figure 4) which persisted even at 24h, reducing blood glucose by 63% ($p<0.001$) from the diabetic control. The more prolonged and increased effect of the extract may be due to the high flavonoid content. The possible explanation for the anti-hyperglycemic effect of *H.cordata* could be the phenol and flavonoid content of the plant. Many studies have reported the hypoglycemic effects of flavonoids on diabetes mellitus [32].

5. Conclusion

Present findings provide an experimental justification to the traditional use of this plant for the management of hyperglycemia. Plant extract exhibited good antioxidant and anti-inflammatory properties which may result in preventing diabetic complications that are generally attributed to excessive oxidative and inflammatory stress during hyperglycemia.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

The study has been performed in accordance with the Institutional Ethics Committee (Animal models) dated 1st October 2018.

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