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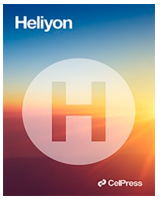

Research article

Optimization of the extraction methods and evaluation of the hypoglycemic effect of *Adhatoda Zeylanica* extracts on artificially induced diabetic mice

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A R T I C L E I N F O

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A B S T R A C T

Synthetic antidiabetic drugs are often associated with various adverse side effects, including hypoglycemia, nausea, gastrointestinal disturbances, headaches, and even liver damage. In contrast, plant-derived natural antidiabetic bioactive compounds typically exhibit lower toxicity and fewer side effects and have been reported to aid effectively in diabetes management. These plant extracts regulate diabetes by restoring pancreatic function, enhancing insulin secretion, inhibiting intestinal glucose absorption, and facilitating insulin dependent metabolism. This study explored four extraction methods, including reflux distillation (RD), ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), and enzyme assisted extraction (EAE) to optimize the yield of crude leaf extract and vasicine from *Adhatoda zeylanica*. RD produced the highest crude extract yield (98.29 g/kg of dried leaf), while MAE was the most effective for vasicine extraction, yielding 2.44 g vasicine per kg dried leaf. High Performance Liquid Chromatography (HPLC) with a diode array detector (DAD) was used to identify and quantify vasicine, a quinazoline alkaloid with known antidiabetic properties. The hypoglycemic effects of leaf extracts were evaluated in alloxan-induced diabetic mice, and the effect of *A. zeylanica* extract was compared to the extracts of *Centella asiatica*, *Allamanda cathartica*, and the standard drug metformin. At a dose of 400 mg/kg body weight (BW), methanolic leaf extracts of *A. zeylanica*, *C. asiatica*, and *A. cathartica* reduced blood glucose level by 78.95 %, 74.50 %, and 70.19 %, respectively, compared to the standard drug metformin, which reduced blood glucose levels by 85.84 %. *A. zeylanica* at 400 mg/kg BW dose and metformin demonstrated statistically similar and significant blood glucose level reduction ($p < 0.001$). Additionally, therapeutic doses of

Abbreviation: Body weight, BW; Diode array detector, DAD; Enzyme assisted extraction, EAE; Estimated marginal means, EMM; Microwave assisted extraction, MAE; Reflux distillation, RD; Reactive oxygen species, ROS; Ultrasound assisted extraction, UAE.

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A. zeylanica leaf extract exhibited low cytotoxicity (cell survival rate >89 %), highlighting its potential as a safe and effective source of antidiabetic agent.

1. Introduction

Diabetes mellitus is a metabolic disorder characterized by insufficient insulin production or ineffective insulin use. The most common forms of the disease are type 1 and type 2 diabetes. In type 1 diabetes, insulin producing pancreatic cells are attacked and destroyed by the immune system, with T-cells targeting beta cells responsible for insulin production [1,2]. The presence of autoantibodies, including glutamic acid decarboxylase 65 (GAD65), tyrosine phosphatases IA-2, insulin, and ZnT8 against islet cell are indication of autoimmune disease, such as type-1 diabetes. The rate at which cells die varies; being fast in babies and slow in adults. The death of cells by the immune system is caused by a mix of genetic and environmental factors that are not well understood. People with type-1 diabetes are also more likely to get other autoimmune diseases like Hashimoto thyroiditis, Graves' disease, and Addison disease [3]. Insulin treatment is necessary for people with type-1 diabetes to maintain normal blood sugar levels throughout their lives. A major contributing factor that can develop diabetes mellitus and other diseases is oxidative stress [4]. Various factors such as obesity, sedentary lifestyle, aging, and eating habit can result in the production of reactive oxygen species (ROS) [5]. In type 2 diabetes, ROS can activate several signaling pathways, including protein kinase C (PKC) and nuclear factor-κβ (NF-κβ), which in turns can inhibit the insulin signaling pathway, leading to insulin resistance in the body [6]. Between two types of diabetes type-2 diabetes is more prevalent and in this type of diabetes the body cannot use produced insulin effectively [7]. According to the International Diabetes Federation (IDF) 537 million adults were living with diabetes in 2021 and the number may rise to 643 million by 2030 and 783 million by 2045. Over 90 % of the people having diabetes have type-2 diabetes [8,9]. There are many types of complications associated with type-2 diabetes, including cardiovascular disease, renal failure, respiratory issues, stroke, infection, cancer, mental illness, and uncontrolled diabetes [10,11]. The mortality rate of patients with type-2 diabetes is higher than the non-diabetes patient [12]. The only way to treat diabetes is to control the amount of sugar in the blood. Type-1 diabetes patients are more likely to take insulin as drug to control their blood glucose level. Type-2 diabetes patients can also take insulin if needed. Different types of insulin are given to people with diabetes based on their health, but it should be used with care because it can cause hypoglycemia, which is a low blood sugar level, headaches, weight gain, flu-like symptoms etc. Insulin secretagogues are another type of drug that lowers blood sugar, like sulfonylureas and meglitinides. Sulfonylureas and meglitinides work in the cells of the pancreas and the liver [13]. Miglitol and acarbose are α-glucosidase inhibitors, whereas biguanides, metformin, and thiazolidinediones are insulin sensitizers. The major target organs of biguanides are the liver and the muscles. Thiazolidinedione acts on adipose tissue and liver, and its molecular target is peroxisome proliferator-activated receptor gamma (PPAR-γ). Both acarbose and miglitol have their molecular target in -glucosidase, and their sites of action are in the intestinal tract [14]. Hypoglycemia, lactic acidosis, irreversible dementia, headache, dizziness, idiosyncratic liver cell damage, and digestive pain are only some of the serious adverse effects of glucose-controlling medications [15]. When given properly, herbal medication and nutraceuticals tend to have fewer and minor side effect compared to synthetic drugs with enhanced efficiency [16–18]. However, improper doses or certain plant extracts may exhibit side effects or toxicity [19]. Therefore, precise dose selection and thorough evaluation of their safety profile are necessary. Natural plant extracts can regulate diabetes through several mechanisms. They can stimulate insulin secretion, enhance insulin sensitivity by activating AMP- activated protein kinase (AMPK) and modulating upstream signaling, inhibit carbohydrate hydrolyzing enzymes like α–amylase and α glucosidase, and by scavenge free radicals, including ROS [20–22]. These effects can contribute to the regulation of blood glucose level and reduction of oxidative stress in diabetic patients, making natural plant extract a promising source for diabetes management.

Nutraceuticals primarily refer to the substances possessing both nutritional and pharmaceutical benefits, are found abundantly in plants. Advances in nutrition science, plant biotechnology and phytotherapy have increased interest in plant-based compounds for various therapeutic applications [23]. The bioactive ingredients of nutraceuticals, also known as phytochemicals, have been shown to possess antioxidant, anti-inflammatory, antibacterial and antiviral properties [24–26]. Today, nutraceuticals are gaining prominence in treating lifestyle-related diseases, including diabetes. Over the years, around 450 plants with hypoglycemic activity have been tested both *in vitro* and *in vivo* [13].

A. zeylanica, C. asiatica, and *A. cathartica,* all these three plants are traditional medicinal herbs abundantly available in Bangladesh and they have hypoglycemic effect [27,28]. The major chemical constituents of *A. zeylanica* belong to quinazoline alkaloidal group. These quinazoline alkaloids are vasicine and vasicinone which are reported for their bronchodilator properties, antioxidant, anti-inflammatory and antidiabetic activities [29,30]. *C. asiatica* is another miraculous medicinal herb considered in Asia and China regions for its beneficial properties in treating various skin conditions, diarrhea, ulcers and diseases of the female genitourinary tract [31]. The third chosen medicinal herb, *A. cathartica* has also been reported to be consisted of various phytochemicals such as phenolic compounds, flavonoids, alkaloids, carbohydrates and phospholipids [32]. Numerous studies have also documented pharmacological activities of *A. cathartica* as a source of traditional medicine with reduced risk of adverse effect [32,33].

This study explores the antidiabetic properties of these three medicinal plants through *in vivo* biochemical assay on alloxan-induced diabetic mice. For this purpose, four different extraction methods were used to optimize the yield of leaf extracts and. *A. zeylanica* leaf extract was used for identification of the bioactive component vasicine, that is responsible for antidiabetic properties using HPLC [34, 35].

2. Materials and method

2.1. Collection of leaves and the preparation of leaf extract

Leaves of *A. zeylanica, C. asiatica,* and *A. cathartica* plants were collected from Botanical Garden, Department of Crop Botany, Bangladesh Agricultural University, Mymensingh, Bangladesh (latitude: 24.7199, longitude: 90.4266) at an altitude of approximately 20 m above sea level [36]. The collected plant samples were identified by Professor (Retired) Md. Mustafizur Rahman, an experienced Botanist at the Department of Crop Botany, Bangladesh Agricultural University, Mymensingh, Bangladesh. The dried herbarium specimens of the plant sample were deposited as voucher (*A. zeylanica*- Md. Mustafizur Rahman V#319, *C. asiatica*- Md. Mustafizur Rahman V#754, *A. cathartica*- Md. Mustafizur Rahman V#173) specimen in the Professor Dr. Arshad Ali Herbarium at the Botanical Garden, Department of Crop Botany, Bangladesh Agricultural University, Mymensingh, Bangladesh. The collected leaves were washed with clean water to remove any impurities and then naturally dried under the sun. The dried leaves were ground into a coarse powder.

2.2. Extraction methods

Four different extraction methods at different conditions were utilized to optimize the amount of leaf extract and vasicine from leaves of *A. zeylanica*. The extraction methods used were traditional RD and other three green extraction methods including UAE, MAE, and EAE [37–40]. In addition to pharmaceutically active compounds, green extraction methods are also applied across various fields and industries. For example, they are used for natural pigments and edible oil extraction in the food industry, and for extracting nanocellulose and natural dyes in the textile industry [41–46]. In this study, only *A. zeylanica* was used for the optimization of these extraction methods. For all the extraction methods, the solid to solvent ratio was 1:25 (w/v). Compared to traditional extraction methods, green extraction consumes less energy, reduces the extraction time and risk of thermal degradation in addition to high yield of extraction [47].

2.2.1. Reflux distillation (RD)

Phytochemicals from the leaves were extracted by reflux distillation extraction method using methanol as solvent in a two neck round bottle flask. A photo of the RD experimental setup is provided in the supporting information (Fig. S1). Reflux distillation extractions were performed for 6 h, 8 h, and 10 h at the boiling point of methanol (64.7 °C). The extracted solution was then filtered through Whatman filter paper and subjected to distillation to concentrate the extract solution as well as solvent recovery. Around 80 % solvent was recovered in distillation process. The concentrated solution was air dried until thick paste like extract was obtained.

2.2.2. Ultrasound assisted extraction (UAE)

Water bath sonication (ROCKER SONER 210 H, Ultrasonic cleaner, 220 V, 50 Hz) was used to extract bioactive compounds from the powdered leaf using methanol as solvent. This method was performed at temperatures of 30 °C, 40 °C, 50 °C, and 60 °C and durations of 30, 50, 70, and 90 min to find the optimum parameters for UAE [48,49].

2.2.3. Microwave assisted extraction (MAE)

The setup for microwave extraction method using NOVA Microwave Oven NV-1101 was same as that of reflux distillation, except for that the heating was achieved through microwave. Methanol was used as the solvent in this process. A photo of the MAE experimental setup is provided in the supporting information (Fig. S2). MAE was performed for 10 min at four different power outputs,400 W, 500 W, 600 W, and 700 W.

2.2.4. Enzyme assisted extraction (EAE)

Pectinase enzyme was used to extract compounds from the leaf at room temperature. Enzyme breaks down the structural integrity of the cell wall of leaf and facilitates the extraction of bioactive compounds [50]. 4 ml of 25 g/L enzyme solution in water was mixed with 250 ml of methanol and 10 g dried leaves, resulting in a solid to solvent ratio of 1:25 (w/v). The mixture was stirred at 200 rpm for 5 different time periods to determine the optimum time of extraction.

2.3. In vivo biochemical assay on alloxan mice

Female Swiss albino mice weighing between 15 and 30 g and aged eight weeks were given an intraperitoneal injection of Alloxan dissolved in saline at a dose of 90 mg/kg BW following a 16-h fast. To mitigate the drug-induced hyperglycemia, the animals were fed overnight. Experiments were conducted on diabetic mice, defined as having a blood sugar level greater than 11.5 mmol/L. Thirty-two rats were divided into eight groups of four at random. The mice in Group 1 were given distilled water as a placebo, whereas those in Group 2 were given standard drug metformin at a dose of 100 mg/kg BW. Methanolic leaf extract of *A. zeylanica* was given to group 3 and 4 at doses of 250 mg/kg BW and 400 mg/kg BW, respectively. Groups 5 and 6, 7 and 8 were also given similar doses of methanolic leaf extracts of *C. asiatica* and *A. cathartica,* respectively. The blood sample was taken from the tail vein of each mouse. Plasma glucose levels were determined using a glucometer (Tyson Bio Evolve-Chu Nan, Taiwan) and glucose strips (Tyson Bio strips). The animal trial was performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and guidelines [51]. All techniques and protocols were approved by the Animal Ethics Committee of the Department of Chemical Engineering of Bangladesh University of Engineering and Technology (BUET/CHE/A2401).

2.4. HPLC analysis of A. zeylanica

High performance liquid chromatography (Thermo-Scientific, USA) equipped with Vanquish quaternary pump C, with Vanquish autosampler (split sampler CT), in combination with Chromeleon software version 7.3.1 was used for analysis. The system utilizes isocratic elution mode, with DAD for peak detection and analysis. Solvents were vacuum filtered through a 0.2 μm filter (Pall Corporation) and analysis was performed on a UMISil C18 column (250 mm × 4.6 mm, 5 μ particle size). The mobile phase used was Acetonitrile: 0.1 M Phosphate buffer: glacial acetic acid (15:85: 1 v/v/v) and pH = 3.9 [52]. The flow rate was 0.8 ml/min. Column temperature was at 26 ° C and the detection wavelength was 300 nm.

United States Pharmacopeia (USP) reference vasicine standard (lot number was R097T0.), obtained from Sigma Aldrich was dissolved in HPLC grade methanol to prepare standard solutions at concentrations of 10 ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm. Sample solutions were prepared by dissolving 10 mg methanolic leaf extract of *A. zeylanica* in 50 ml methanol, sonicated for 5 min and the volume of the solution was made 100 ml to get a 100 ppm sample solution. 10 μl of each standard and sample solutions were injected to HPLC column [53]. The run time for each injection was 15 min.

For quantifying vasicine, an analytical calibration curve was established using five standard vasicine concentrations. Single measurements were taken for the samples, and the amount of vasicine in the extracts was quantified using this calibration curve. The calibration curve demonstrated a strong correlation with R^2 value of 0.9997. The limits of detection (LOD) and quantification (LOQ) were determined to be 0.77 ppm and 2.56 ppm respectively, using the following equations:

LOD = (3.3 × Residual Standard Deviation)/(Slope of the calibration curve)

LOQ = (10 × Residual Standard Deviation)/(Slope of the calibration curve)

2.5. Cytotoxicity evaluation of methanolic leaf extract of A. zeylanica

The cytotoxicity of the methanolic leaf extract of *A. zeylanica* was performed in Vero cell line, which consists of kidney epithelial cells extracted from an African green monkey. The cells were maintained in Dulbecco's Modified Eagles' Medium (DMEM) containing 1 % penicillin-streptomycin (1:1) and 0.2 % gentamycin, and 10 % fetal bovine serum (FBS).

The cells (1.5×10^4/100 μl) were seeded in a 96-well plate and incubated at 37 °C in a 5 % CO_2 atmosphere. After 24 h of incubation, 25 μl of the methanolic leaf extract at concentrations of 100, 200, 400, and 800 mg/L were added to each well. Following further 48 h of incubation, cell viability was assessed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, USA).

2.6. Statistical analysis

Statistical analysis of the *in vivo* mouse trial data was conducted using IBM SPSS Statistics version 30.0.0. To evaluate the effects of administered treatments (control, standard, and plant extracts doses) on the blood glucose levels over time, a repeated measures analysis of variance (ANOVA) was performed separately for each trial to examine the interactions between blood glucose level at different time and the administered doses, accounting for within subject correlations. Mauchly's test was used to check the sphericity assumption, which was not violated in this analysis.

Pairwise comparisons between treatment groups at each time point (0, 60, 120, 180, and 240 min) were conducted using estimated marginal means (EMM) with Bonferroni confidence interval adjustment. Additionally, one-way ANOVA were performed at each time point for each trial followed by Tukey's Honest Significant Difference (HSD) post-hoc test to adjust for multiple comparisons. This one-way ANOVA with Tukey's HSD post-hoc test was also applied to other data sets within the study where it is applicable.

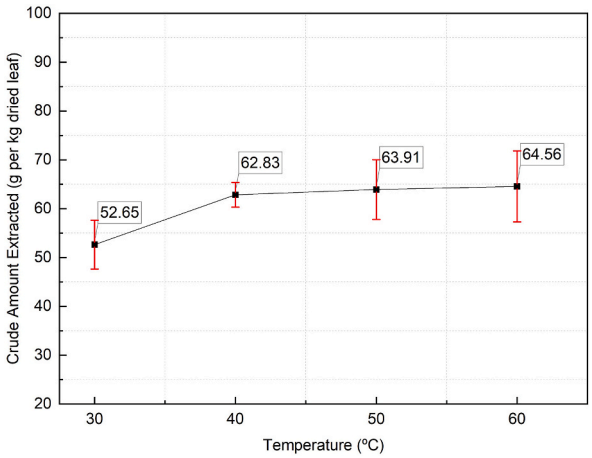

Fig. 1. Effect of temperature on the amount extracted by ultrasound assisted extraction.

Results of the mouse trial data are presented as mean ± standard error (SE) of the mean, and statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Maximization of crude leaf extract from A. zeylanica

Four different types of extraction methods including EAE, MAE, UAE, and RD were compared in this study. As UAE has two primary parameters, time and temperature, initially the optimum temperature of UAE was identified.

Fig. 1 shows the variation in the amount of extract obtained by UAE from *A. zeylanica* leaf at different temperatures (30 °C, 40 °C, 50 °C, and 60 °C), with an extraction time of 70 min. Although 60 °C provided average highest yield, statistical analysis using Tukey HSD test indicated no significant differences in amount extracted for the tested temperatures ($p > 0.05$). This analysis suggests that within the tested range, temperature variations do not significantly influence the extraction amounts. Nevertheless, for consistency, 60 °C was selected as the extraction temperature for subsequent UAE experiments with varying extraction time. The maximum operating temperature for the sonication was also taken to be 60 °C as the boiling point of methanol is 64.7 °C and the sonication experiment was designed as open system sonication. In addition, at higher temperature cushioning effect is observed in sonication method. Though at higher temperature more bubbles will be formed, the elevated temperature would create vapor filled bubbles that would decrease the effect of cavitation, and this cushioning effect would level out the yield of leaf extract [54].

Fig. 2 shows the variation in amount of crude leaf extract obtained with the four extraction methods. Among different hours of extraction for EAE, 3 h extraction resulted in the highest amount of crude leaf extract. MAE at 700 W power output yielded the highest amount of crude leaf extract. UAE was performed at different temperatures and time and the optimum temperature and time was found to be at 60 °C and 70 min. At this optimum condition UAE yielded the highest amount of crude extract. Extraction from the leaves of *A. zeylanica* using reflux distillation was performed at 6 h, 8 h, and 10 h. The 10 h extraction resulted in the highest amount of crude leaf extract that was 98.29 g crude extract per kg dried leaf. Thus, among the four extraction methods and conditions RD 10 h produced the highest yield of crude leaf extract. The increased yield is due to the enhanced mass transfer at elevated solvent temperature for a long period of time.

3.2. Assessment of vasicine in A. zeylanica

The crude leaf extract of *A. zeylanica* contained different kinds of inorganics, biopolymers, primary or basic metabolites and secondary metabolites [55]. It is the secondary metabolites those are normally responsible for hypoglycemic effect [56]. The secondary metabolites are quinazoline alkaloids, namely vasicine, vasicinone, vasicol, and peganine as major constituents. Other minor alkaloids include adhatodine, vasicinol and vasicinolone [57]. Vasicine is reported to be one of the prominent components in the entire plant [58]. Vasicine has antidiabetic activity because of its significant inhibitory effects against α-glucosidase enzyme [35,59]. The presence and quantity of vasicine was determined in the methanolic leaf extract of *A. zeylanica* by HPLC analysis.

The same retention time for the vasicine standard and *A. zeylanica* leaf extract in Fig. 3 confirmed the identification of vasicine in the leaf extract of *A. zeylanica*.

Quantification of vasicine in the extract was also performed by HPLC analysis as shown in Fig. 4. Among different hours of extraction for EAE, 3 h enzyme assisted extraction resulted in highest amount of vasicine. The continuous reduction in vasicine content

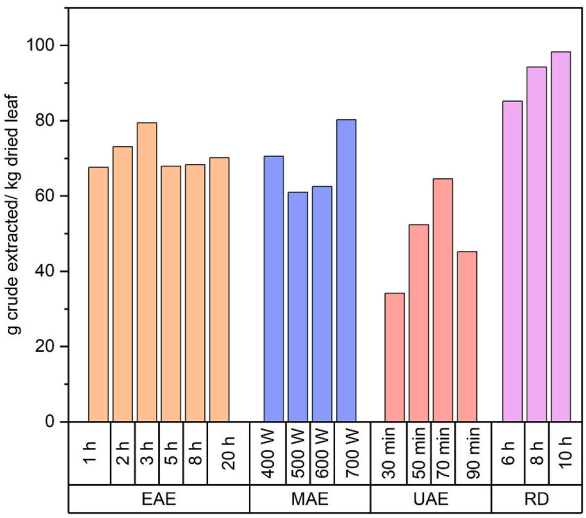

Fig. 2. Amount of crude extract from dried *A. zeylanica* leaf at different conditions of four extraction methods.

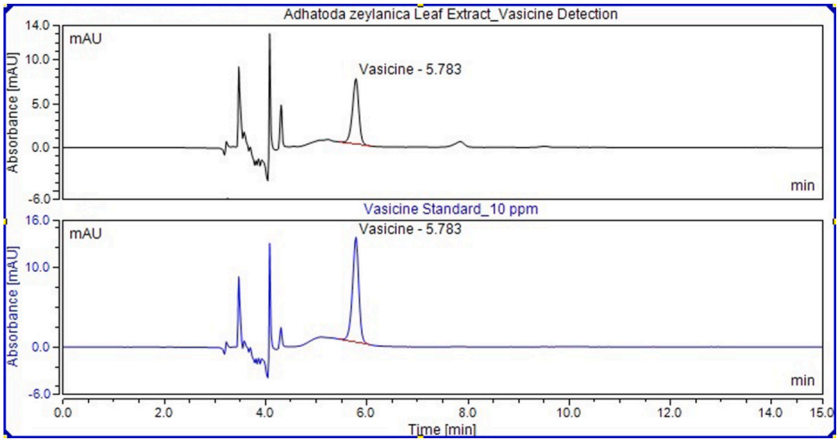

Fig. 3. HPLC chromatogram of *A. zeylanica* leaf extract and vasicine standard.

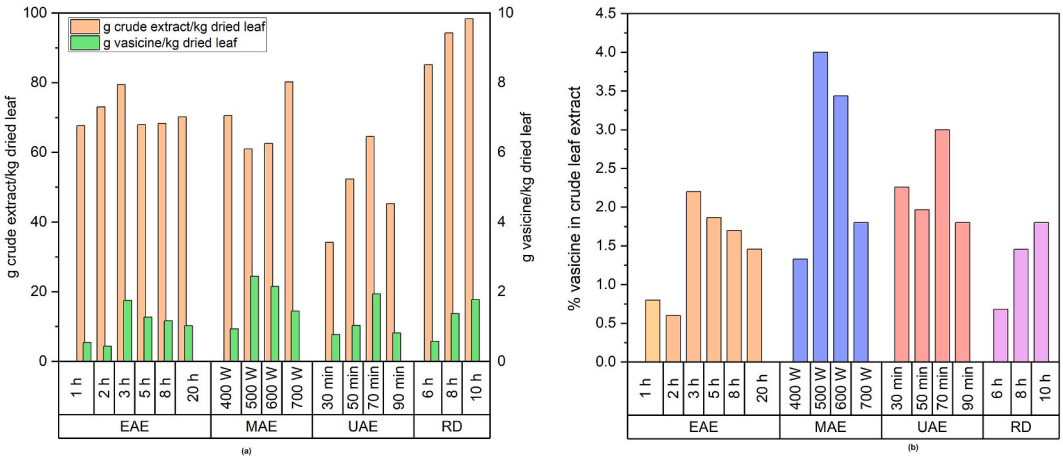

Fig. 4. (a) Amount of vasicine compared to the amount of crude extract and (b) % of vasicine in the crude extract from dried *A. zeylanica* leaf at different conditions of four extraction methods.

above 3 h of EAE is because enzymes not only hydrolyze the cell wall of leaves but also can hydrolyze active compounds into other compounds if treated for prolonged period of time. Among the different power output for MAE, at 500 W power yield of vasicine was highest. Above 500 W power though crude extract yield was higher but the vasicine extract was lower. This lower vasicine content at higher power is due to the thermal degradation of vasicine as internal temperature of the solid matrix increases with increasing power [60,61]. The yield of vasicine in UAE method was largest at the optimum condition of UAE, that was 70 min and 60 °C. Lesser yield of vasicine at 90 min of UAE is because prolonged ultrasound treatment can degrade active compounds [62]. Fig. 4 (a) also shows that at UAE 90 min the amount of total crude extract was also less than that of 70 min. The decrease in the amount of crude extract might be attributed to the formation of insoluble compounds resulting from the degradation of the extracted active compounds during the prolonged extraction process. Reflux distillation extraction of leaf of *A. zeylanica* was performed at different times, varying from 6 h to 10 h, and the 10 h extraction resulted in highest amount of crude leaf extract as well as vasicine.

The comparative data of the four different extraction methods and conditions from Fig. 4 (a) shows that 10 h reflux distillation yielded the highest 98.29 g crude extract per kg of dried *A. zeylanica* leaf. But the percentage of vasicine in the crude extract from Fig. 4 (b) shows the MAE 500 W extract had the highest percentage of vasicine in the crude extract and the extraction time was just 10 min. The higher amount of vasicine in MAE with a shorter extraction time is attributed to the distinct mechanism of extraction by MAE and RD. In MAE accelerated extraction takes place by synergistic combination of same direction of heat and mass transfer [63]. In case of MAE, electromagnetic radiation instantly raises the internal temperature of the solid matrix, leading to rapid cell rupture and extraction of phytochemicals within short frame [61]. With optimum power output and short time of extraction, vasicine degradation is minimized. In case of RD, conduction and convection heat transfer raised the solid matrix temperature slowly up to the boiling point of solvent methanol. The combination of solvent penetration, solvent-solute interaction and diffusion resulted in the extraction of phytochemicals with a longer time period. The constant high temperature may contribute to the degradation of a portion of vasicine during the extraction process. This degradation resulted in a decrease in the concentration of vasicine in the crude leaf extract obtained

through RD compared to that obtained through MAE, as shown in Fig. 4.

The findings from Fig. 4 indicate that the most effective method and conditions for extracting vasicine from *A. zeylanica* leaf extract were achieved with MAE at 500 W power output with an extraction time of 10 min.

3.3. Cytotoxicity evaluation

The cytotoxicity evaluation of methanolic leaf extract of *A. zeylanica* was performed in Vero cell line. Survival rates for cells treated with four different concentrations of extract over 48 h are summarized in Table 1.

The survival rates in Table 1 suggest that none of the tested doses of the methanolic leaf extract of *A. zeylanica* had significant cytotoxic effects on Vero cells, with survival rates ranging from 89.68 % to 94.91 % (see Table 1). Even at the highest concentration of 800 mg/L, the extract showed minimal cytotoxicity with survival rate of 90.45 %, indicating that the cell viability was maintained across all tested doses. Images of the treated cell lines are provided in Fig. S3 (Supporting Information). These cytotoxic results are consistent with similar study on *A. zeylanica* extract in Vero cells, which also reported high cell viability at comparable concentration [64]. The low cytotoxic effect of *A. zeylanica* leaf extract observed in Vero cells implies its potential for further therapeutic development.

3.4. Antidiabetic effect of the leaf extracts on blood glucose level

Three sets of animal trials were conducted to study the antidiabetic effect of leaf extracts. The results in Table 2 showed that the introduction of the leaf extracts and metformin reduced blood glucose levels significantly compared to control group during the 4-h study period. Methanolic leaf extract used in the animal trial was obtained by RD 10 h method as this condition yielded the highest amount of crude leaf extract. To compare the performance of the *A.* zeylanica extracts, distilled water was used as placebo control, metformin was used as positive control and extracts of *C. asiatica* and *A. cathartica* were used for relative differences as the latter two extracts were also reported to have antidiabetic properties to some extent.

Both Pairwise comparisons using EMM with Bonferroni confidence interval adjustment and independent one-way ANOVA with Tukey's HSD revealed significant differences between the control group, and both the standard metformin and *A. zeylanica* 400 mg/kg BW dose groups at 180 and 240 min. Additionally, *C. asiatica* and showed significant differences ($p < 0.001$) from the control group at 240 min in trials 1 and 3, and *A. cathartica* in trials 3. However, only *A. zeylanica* 400 mg/kg BW dose consistently showed $p < 0.001$ at 240 min across all the three trials, similar to the standard drug metformin.

Fig. S4 (Supporting Information) summarizes the result of three trials and demonstrates different levels of blood glucose reduction achieved by the doses of the three plant leaf extracts, that are significantly greater than those of control group. Among the three plants, *A. zeylanica* showed the highest antidiabetic effect, comparable to that of standard drug metformin, aligning with the statistical analysis results.

Fig. 5a and b illustrate the effects of two different doses of *A. zeylanica* leaf extract on blood glucose level reduction compared to the standard drug metformin. Fig. 5a presents the absolute reduction in blood glucose level, while Fig. 5b presents percentage reduction, which was calculated by adjusting the expected reductions observed in the control group (distilled water, shown in Fig. 5a), isolating the effect of the leaf extracts alone. The doses of crude leaf extract, 400 mg/kg BW and 200 mg/kg BW, contained vasicine at amount of 7.2 mg/kg BW and 3.6 mg/kg BW respectively. Fig. 5b shows that 400 mg/kg BW and 200 mg/kg BW doses reduced blood glucose level by 78.95 % and 70.19 % respectively, both of which are remarkably close to the reduction achieved by standard drug, which was 85.84 %. Compared to the standard drug dose of 100 mg/kg BW, a smaller amount of vasicine form *A. zeylanica* leaf extract was almost as effective in reducing blood glucose level. Although other alkaloids might have an individual or synergistic effect on reducing the blood glucose level, vasicine makes up the vast majority of the total alkaloids in the leaf [65]. Our study showed that *A. zeylanica* had enhanced efficiency in reducing blood glucose levels, achieving 78.95 % reduction at 400 mg/kg BW dose. This reduction was greater compared to similar studies with different other plant extracts. For instance, a 200 mg/kg BW dose of *Scoparia dulcis* plant extract reduced blood glucose level by 49.17 %, a 400 mg/kg BW dose of *Juglans regia* (Walnut) leaf extract reduced blood glucose level by 20.1 %, and *Momordica charantia* fruit extract at 500 mg/kg BW dose reduced blood glucose level in alloxan-induce diabetic mice by 25.51 % [15,66,67]. Additionally, another study reported a significant blood glucose level reduction by *A. zeylanica* leaf extract compared to standard drug, that is consistent with our findings [27].

In the present study, a 400 mg/kg BW dose of *C. asiatica* and *A. cathartica* leaf extracts reduced blood glucose levels of alloxan induced diabetic mice by 74.50 % and 67.52 % respectively (Fig. S4). Notably, the same dose of *A. zeylanica* leaf extract resulted in

Table 1
Survival rate of Vero cells after 48 h treatment with methanolic leaf extract of *A. zeylanica*.

Dose (mg/L)	Survival rate (%)
Control	100.00
100	94.91
200	94.64
400	89.68
800	90.45

Table 2
Hypoglycemic effect of methanolic leaf extracts of *A. zeylanica, C. asiatica and A. cathartica* on animal trial 1, 2, and 3.

Animal Group			Control (Distilled Water)	Standard (Metformin)	*A. zeylanica*		*C. asiatica*		*A. cathartica*	
Dose/kg body weight			10 ml	100 mg	250 mg	400 mg	250 mg	400 mg	250 mg	400 mg
Plasma glucose level (mmol/L)	Trial-1	0 min	24.15 ±1.29	25.43 ±0.94	25.30 ±0.44	24.38 ±0.40	23.64 ±0.60	26.40 ±0.80	23.90 ±0.82	24.83 ±0.58
		60 min	27.13 ±1.33	31.15* ±0.52	28.65 ±0.79	28.78 ±0.49	25.25 ±0.39	30.90* ±0.61	27.10 ±0.47	27.95 ±0.58
		120 min	25.48 ±1.21	21.23* ±1.68	25.38 ±0.59	22.93 ±0.53	22.13 ±0.41	25.45 ±0.27	25.10 ±0.50	25.53 ±0.44
		180 min	23.95 ±0.99	16.15** ±0.85	22.15 ±0.42	18.70** ±0.53	19.68* ±0.41	21.68 ±0.73	23.05 ±0.61	22.73 ±0.36
		240 min	23.50 ±1.24	13.20** ±0.74	18.90** ±0.52	14.45** ±0.48	17.38** ±0.41	18.55** ±0.41	21.70 ±0.50	21.25 ±0.44
	Trial-2	0 min	21.55 ±0.42	24.43 ±0.33	21.23 ±0.34	21.35 ±0.34	19.53 ±1.13	23.25 ±3.14	18.80 ±1.11	20.38 ±0.33
		60 min	20.45 ±0.46	21.90 ±0.68	17.93 ±0.58	17.13 ±0.76	18.88 ±1.20	21.45 ±2.26	18.15 ±1.23	19.28 ±0.36
		120 min	19.90 ±0.24	18.98 ±0.58	16.50 ±0.48	15.38* ±0.98	17.48 ±1.02	20.08 ±1.66	17.00 ±1.11	17.33 ±0.51
		180 min	19.45 ±0.25	16.15 ±0.85	15.63 ±0.26	14.78* ±0.93	15.53 ±1.22	19.18 ±1.54	16.30 ±1.11	15.38 ±0.38
		240 min	19.03 ±0.26	10.45** ±0.31	14.80* ±0.38	12.30** ±0.56	14.80* ±1.14	15.93 ±0.86	14.68* ±0.64	14.25* ±0.64
	Trial-3	0 min	21.05 ±0.35	26.58** ±0.47	22.45 ±1.40	21.70 ±0.87	20.53 ±0.40	21.75 ±0.76	21.23 ±0.58	21.48 ±0.51
		60 min	19.70 ±0.23	21.65 ±0.52	20.88 ±1.34	19.63 ±0.85	18.70 ±0.80	19.45 ±0.79	19.15 ±0.42	19.28 ±0.35
		120 min	19.15 ±0.23	17.98 ±0.58	18.68 ±1.17	17.20 ±0.78	17.35 ±0.44	16.58 ±0.60	17.15 ±0.51	16.00 ±0.83
		180 min	18.28 ±0.41	14.65* ±0.63	16.93 ±0.64	14.48* ±0.79	14.93* ±0.66	15.43 ±0.72	16.00 ±0.54	14.40* ±0.80
		240 min	17.90 ±0.40	9.70** ±0.19	14.65* ±0.53	10.75** ±0.67	13.80** ±0.70	12.43** ±0.74	15.05* ±0.46	13.23** ±0.68

Note: * indicates $p < 0.05$, and ** indicates $p < 0.001$ compared to control group.
The statistical analysis using repeated measure ANOVA demonstrated a significant interaction between time and treatment group ($p < 0.001$), indicating that the effects of administered doses (control, standard, and plant extract doses) on blood glucose level varied across time points.

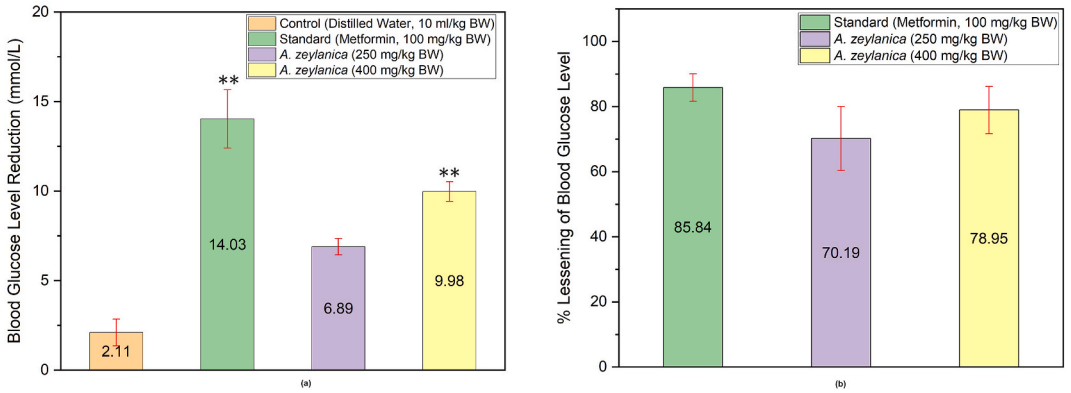

Fig. 5. (a) Average glucose level reduction (mmol/L) with standard error (** indicates $p < 0.001$ compared to control group) and (b) Average of the percentage glucose level reduction of standard drug and *A. zeylanica* samples by deducting the effect of control distilled water.

78.95 % reduction in blood glucose level. The comparison of different plant extracts with the results of the present study suggests that *A. zeylanica* leaf extract has significant potential as an effective antidiabetic drug. The antidiabetic effect of *A. zeylanica* can be attributed to different mechanisms. Its antioxidant properties may mitigate oxidative stress and enhance insulin sensitivity by suppressing the activation of NF-κβ, potentially reversing the inhibition of insulin signaling pathway [6,58,68–70]. Additionally, *A. zeylanica* extract can inhibit carbohydrate hydrolyzing enzymes, such as α-glucosidase and α-amylase, which can regulate blood glucose level by delaying carbohydrate digestion [35,59,71–73]. Furthermore, the leaf extract of *A. zeylanica* can enhance cellular glucose uptake by activating phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) and AMPK pathways independently of

insulin, suggesting its potential application for individuals with insulin-resistance [74]. In addition to *A. zeylanica*, both *C. asiatica* and *A. cathartica* exhibited hypoglycemic effects. Secondary metabolites present in *C. asiatica* are mainly triterpenes [75] and they have antidiabetic properties [76]. *A. cathartica* consists of alkaloids, flavonoids and phenolic compounds [77]. All of these secondary metabolite groups have antidiabetic effects [59,78,79]. *C. asiatica* may help control the blood glucose level through its antioxidant properties, enhanced glucose storage, and lowered glucose release in the liver [28,75,80]. Similarly, *A. cathartica* have antioxidant properties and can inhibit carbohydrate hydrolyzing enzymes, which could contribute to lowering the blood glucose level [77,81,82]. Overall, *A. zeylanica* demonstrates significant potential in diabetes management through multiple mechanisms. Additionally, the hypoglycemic effects of *C. asiatica* and *A. cathartica* along with their potential mechanisms of action further support their therapeutic value diabetes management.

4. Conclusion

This study demonstrated that MAE at 500 W was the most effective method to isolate vasicine from *A. zeylanica* leaf extract, achieving the highest yield of this metabolite. As a greener extraction technique, MAE enhances efficiency, reduces processing time, and maintains energy efficiency, making it an environmentally sustainable option.

The *in vivo* trials confirmed the significant hypoglycemic effects of the plant extracts, with *A. zeylanica* exhibiting the highest efficacy, compared to the standard drug. Three independent trials with comprehensive statistical analysis highlighted the reliability and reproducibility of these results. However, a limitation of this study is the use of single measurements for vasicine quantification across different extraction methods and conditions, which may introduce variability.

Future studies may focus on investigating the specific mechanisms of underlying hypoglycemic effects by these plant extracts. Elucidating the pathways of crude leaf extracts and the roles of individual secondary metabolites could provide insights into their individual and synergistic contribution to the overall hypoglycemic effect.

Although the therapeutic doses of *A. zeylanica* showed low cytotoxic effects on Vero cell lines, future research could further examine the cytotoxic effects of other two plant extracts given their significant hypoglycemic properties. To expand the safety profile of these leaf extracts, further *in vitro* studies in additional cell lines and subsequent *in vivo* study should be conducted. Successful preclinical research could support formulation development, paving the way of clinical trials and the potential use of plant-based phytochemicals as commercial antidiabetic agents.

CRediT authorship contribution statement

Md Fahim Ahmed: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation. **Khalid Hasan Raby:** Writing – original draft, Project administration, Methodology, Investigation. **Nishat Tasnim:** Project administration, Methodology, Investigation. **Md Tariful Islam:** Project administration, Methodology, Investigation. **Mahbub Chowdhury:** Methodology, Investigation. **Zarin Tasnim Juthi:** Methodology, Investigation. **Md Ashik Mia:** Resources, Methodology. **Lubna Jahan:** Supervision, Project administration. **A.K.M. Zakir Hossain:** Resources, Conceptualization. **Shoeb Ahmed:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Ethical statement

All animal experiments complied with ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. The study was conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, its associated guidelines, and the EU Directive 2010/63 for the protection of animals used for scientific purposes. The experimental procedures were approved by the Animal Ethics Committee of the Department of Chemical Engineering, Bangladesh University of Engineering and Technology (BUET/CHE/A2401). Female Swiss albino mice were used in in this study following the NIH (National Research Council) Guide for the Care and Use of Laboratory Animals.

Data availability statement

The majority of data generated during this study are included in the manuscript and supplementary materials. Data not included are available upon reasonable request from the corresponding author.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e41627.

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