

BRASSICA OLERACEA L. VAR. ITALICA PLENCK AND CASSIA ABSUS L. EXTRACTS REDUCE OXIDATIVE STRESS, ALLOXAN INDUCED HYPERGLYCEMIA AND INDICES OF DIABETIC COMPLICATIONS

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Abstract

The nature's endowment of medicinal plants in successful management of diabetes necessitates their further exploration. Therefore, the present study was designed to comprehend ameliorating role of *Brassica oleracea* var. *italica* (BO) and *Cassia absus* (CA) in oxidative stress, hyperglycemia and indices of diabetic complications. Among all the extracts of BO and CA, aqueous extract was the most proficient in terms of extract recovery (9.0 and 10.2%) and DPPH radical scavenging efficiency ($IC_{50} = 11.90 \pm 1.70$ and 8.26 ± 1.20 μ g/ml) respectively. Maximum phenolic content [BO = 184.0 ± 0.17 and CA = 406.7 ± 0.08 μ g gallic acid equivalent/mg extract (E)], flavonoid content (BO = 160.9 ± 0.1 and CA = 361.9 ± 0.09 μ g quercetin equivalent/mg E) and total antioxidant capacity (BO = 223.7 ± 0.20 and CA = 257.2 ± 3.30 μ g ascorbic acid equivalent/mg E) was recorded in their ethanol extract. Highest reducing power potential was quantified in BO ethanol and CA aqueous extracts as 427.9 ± 0.10 and 480.0 ± 2.10 μ g ascorbic acid equivalent/mg E respectively. Brine shrimp assay expounded petroleum ether extract of BO and CA to have some cytotoxicity ($LC_{50} = 200 \pm 2.3$ and 86.6 ± 3.1 μ g/ml respectively). *In vivo* studies established their aqueous extract as proficient in reducing the serum glucose (BO = 142.3 ± 7.10 and CA = 161.5 ± 4.40 mg/dl at day 21) as well as blood cholesterol, ALT and urea levels. Findings of the present study prospects BO and CA a useful treatment of diabetes and its escorting complications.

Key words: *Brassica oleracea*, *Cassia absus*, Hyperglycemia, Diabetes mellitus, Oxidative stress.

Introduction

Diabetes mellitus (DM), a multifactorial disease, is characterized by impaired insulin secretion or its action for glucose regulation (Zimmet *et al.*, 2001). The wrecking effects of DM qualifies it as an ailment of foremost public health concern. Epidemiological data revealed it as the seventh leading cause of death worldwide and is estimated to be the principal cause of morbidity and mortality within the next 25 years, especially in Asia and Africa. The disease if neglected can lead to the problems like cardiovascular anomalies, nerve damage and nephropathies (Ojiako *et al.*, 2016). The curbs allied with contemporary antidiabetics such as adverse effects, high cost of treatment and development of resistance have encouraged complementary therapy as more efficient alternative for the management of diabetes epidemic. Moreover, scientific literature shows that medicinal plants used in traditional systems of medicine as hypoglycemic agents have also proved experimental or clinical antidiabetic potential (Pandey *et al.*, 2011).

Numerous hypotheses about the onset and progression of diabetic complications have been proposed by a number of researchers, but one of the most important is the role of reactive oxygen species (ROS). ROS can directly impose molecular as well as cellular damage through activation of a cascade of stress-sensitive pathways and ultimately result in chronic complications of the disease (Khan *et al.*, 2015). The origin of some of the contemporary treatments from nature's hub such as voglibose, acarbose and miglitol highlights the need to further explore other natural sources for the provision of better treatment options (Newman & Cragg, 2012). Ethnopharmacological approach is

particularly a valuable strategy to limit the gigantic multiplicity of possible leads to more valuable hits. Therefore, *Brassica oleracea* L. var. *italica* Plenck. (Family: Brassicaceae) and *Cassia absus* L. (Family: Fabaceae, subfamily: Caesalpiniaceae), the two important antidiabetic folklores have been evaluated to determine their antihyperglycemic role as well as their effects on DM associated anomalies such as oxidative stress, disorders in lipid metabolism, kidney and liver damage have also been studied. *Brassica oleracea* var. *italica* (BO) commonly known as broccoli is an Italian native plant and a cool weather crop. It is cultivated ubiquitously these days (Mukherjee & Mishra, 2012). BO is mostly used as a food source and is reported to harbor a number of bioactive compounds with anticancer, anti-arthritis and skin healing capabilities (Lehman, 2014). *Cassia absus* (CA) locally known as Chaksuis an Asian native herb or undershrub and is widely distributed in tropical areas of the world especially South Asia and is used locally for the management of various conditions such as snake bite, asthma, bronchitis, for cough and as detoxifier and purgative (Aftab *et al.*, 1996). Most common phytochemicals reported from CA are alkaloids (chaksine and isochaksine), galactomannans, oils, fatty acids, sterols, glycosides, gums and resins (Pandya *et al.*, 2010).

Despite of the several ethnomedicinal claims, scientific profiling of bioactivity of the subject plants is still in paucity. To the best of our knowledge this is the first comprehensive report of CA seed extracts exploring their antioxidant potential, cytotoxic profile, *In vitro* antidiabetic and *In vivo* antihyperglycemic effect. Furthermore, the antihyperglycemic effect of BO extracts employing solvents of escalating polarities in alloxan induced diabetic model has not been reported previously.

Materials and Methods

Solvents and reagents: All solvents and reagents employed in the current study were of analytical grade. Organic solvents included chloroform, petroleum ether (pet-ether), ethanol, dimethyl sulfoxide (DMSO), Folin-Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), sea salt, doxorubicin and alloxan monohydrate were purchased from Sigma-Aldrich (Germany). Phosphate buffer, gallic acid, quercetin, potassium acetate, aluminium chloride, ascorbic acid, aluminium molybdate, potassium ferricyanide, trichloroacetic acid, ferric chloride and alpha amylase enzyme were acquired from Merck (Darmstadt, Germany). Brine shrimp eggs were purchased from Ocean Star Int. (USA).

Collection and extraction: The flowering heads of BO and CA seeds were collected in August and September, 2014 from Islamabad region, Pakistan and were authenticated by Prof. Dr. Rizwana A. Qureshi, Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan. Voucher specimens PHM-492 (*Brassica oleracea* var. *italica*) and PHM-493 (*Cassia absus*) were deposited in the herbarium of medicinal plants, Department of Pharmacy, Quaid-i-Azam University, Islamabad. The plant parts were washed, shade dried and grounded to a fine powder. Accurately weighed 250 g of each powdered plant material was subjected to sonication aided maceration in 1000 ml of water, ethanol, chloroform and petroleum ether (pet-ether) for 24 hours at room temperature in separate Erlenmeyer flasks. The Same procedure was used to extract the marc thrice. The extracts were combined, concentrated by vacuum evaporation in rotary evaporator (Buchi, Switzerland) and dried in vacuum oven (Yamato, Japan) at 45°C to obtain final crude extracts. Percentage yields were calculated and dried extracts were stored at -20°C for further testing. The percentage yield of the extracts was determined gravimetrically using the following formula:

$$\text{Extract recovery (\%)} = (A / B) \times 100$$

where, A = Weight of crude extract obtained after drying and B = Weight of powdered plant material taken for each extraction i.e., 250 g (Fatima *et al.*, 2015).

Phytochemical analysis

Estimation of total phenolic content (TPC): Folin-Ciocalteu (FC) reagent protocol was employed for the assessment of total phenols (Zafar *et al.*, 2016). Concisely, 20 µl from sample stock solution and FC (90 µl) reagent was kept for 5 min at room temperature trailed by the transfer of sodium carbonate solution (90 µl) in 96 well plate. Absorbance of the assay plate was measured at 630 nm using microplate reader (Biotech USA, microplate reader Elx 800). A calibration curve ($y = 0.0136x + 0.0845$, $R^2 = 0.9861$) of gallic acid (6.25-50 µg/ml) as positive standard was attained and the protocol was executed as triplicate analysis. The results are stated as µg gallic acid equivalent per mg extract (µg GAE/mg E).

Determination of total flavonoid content (TFC):

Aluminum chloride colorimetric method was employed for the estimation of total flavonoid content of the test samples (Khan *et al.*, 2015). Concisely, 20 µl from each test extract stock solution (4 mg/ml DMSO), 10 µl of aluminum chloride (10% w/v in H₂O), 10 µl of 1.0 M potassium acetate and 160 µl of distilled water were added in 96 well plate which was kept at room temperature for 30 min. The absorbance of the plate was recorded at 415 nm using microplate reader (Biotech USA, microplate reader Elx 800). The calibration curve equation obtained for quercetin (2.5-40 µg/ml) as positive control was $y = 0.0268x + 0.00764$ ($R^2 = 0.9851$) and the resultant flavonoid content is articulated as µg of quercetin equivalent/mg of extract (µg QE/mg E).

Biological evaluation

In vitro assays

Free radical scavenging activity (FRSA): The FRSA of the crude extracts was monitored by measuring their capability to satiate the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical (Ihtisham *et al.*, 2013). Briefly, 20 µl of each test sample at a final concentration of 200 µg/ml, was mixed with 180 µl of DPPH solution (9.2 mg/100 ml methanol) in 96 well plate. After incubation for 30 min at 37°C, the absorbance of the plate was measured at 517 nm. Percent radical scavenging activity (%FRSA) was calculated by using the formula:

$$\% \text{ FRSA} = (1 - A_b / A_n) \times 100$$

where A_b is the absorbance of test extracts, whereas A_n is the absorbance of negative control containing the DMSO instead of sample (Fatima *et al.*, 2015). Ascorbic acid was used as positive control and the assay was performed in triplicate. Afterwards samples with significant radical scavenging efficiency (> 50%) were further tested at lower concentrations of 66.66, 22.22 and 7.41 µg/ml and IC₅₀ was also calculated by using Table curve 2D v5.01 software.

Total reducing power (TRP) determination: The total reducing power (TRP) of both the plants was determined according to the ferric reducing power assay as described previously (Jafri *et al.*, 2014). Briefly, an aliquot of 200 µl of test extracts from 4 mg/ml stock solution, 400 µl each of phosphate buffer (0.2 mol/l, pH 6.6) and potassium ferricyanide (1% w/v in H₂O) were mixed together in Eppendorf tubes. The aforementioned mixture was incubated for 20 min at 50°C followed by addition of 400 µl of trichloroacetic acid (10% w/v in H₂O). Afterwards, it was centrifuged at 3000 rpm at room temperature for 10 min. The upper layer of solution (500 µl) was mixed with distilled water (500 µl) and 100 µl of FeCl₃ (0.1% w/v in H₂O). From this mixture, 200 µl was transferred to 96 well plate and the absorbance of the reaction mixture was measured at 630 nm. Blank was prepared by substituting 200 µl of DMSO instead of test sample to the aforementioned reaction mixture. A calibration curve ($y = 0.038x + 0.7484$, $R^2 = 0.9967$) of ascorbic acid as positive

control was drawn at final concentrations of 100, 50, 25, 12.5, 6.25, 3.12 µg/ml and the resultant reducing power is expressed as µg of ascorbic acid equivalent/mg of extract (µg AAE/mg E). The assay was performed in triplicate.

Total antioxidant capacity assay (TAC): Total antioxidant capacity was evaluated by employing phosphomolybdenum based colorimetric assay and the results were expressed as µg ascorbic acid equivalent per mg of extract (µg AAE/mg E) as described previously (Haq *et al.*, 2012). An aliquot of 0.1 ml of each test sample (4 mg/ml DMSO) and positive control (at final concentrations of 100-3.12 µg/ml) was mixed with 0.9 ml of reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate solution in H₂O) in Eppendorf tubes. Blank was prepared by mixing 0.9 ml of reagent solution and 0.1 ml of DMSO without extract. The tubes were incubated in water bath for 90 min at 95°C and were cooled to room temperature from which 200 µl was shifted to 96 well plate and the absorbance was measured at 630 nm. A calibration curve ($y = 0.0212x + 0.0926$, $R^2 = 0.9913$) of ascorbic acid was obtained and the experiment was performed in triplicate.

Brine shrimp lethality assay: A 24 hour lethality assay was executed using brine shrimp (*Artemia salina* Linnaeus) larvae in a 96 well plate in accordance with the formerly described procedure (Nasir *et al.*, 2017). *A. salina* eggs (Ocean Star 90, USA) were hatched by incubating for 24-48 hour in simulated sea water (38 g/l sea salt supplemented with 6 mg/l dried yeast) in a precisely designed two compartment plastic tray kept under warmth (30-32°C) and light. Ten nauplii were reaped using Pasteur pipette and were transferred to 96 well plate. Corresponding volume of each extract comprising ≤ 1% DMSO in sea water at final doses of 500, 250, 125 and 62.5 µg/ml was instilled to the wells containing shrimp larvae and sea water. Positive (doxorubicin) and negative (1% DMSO in sea water) control wells were included to exclude false positive and negative results. After 24 hours incubation, the surviving nauplii were counted and the percentage of deaths was determined. Median lethal concentration (LC₅₀) of the test extracts with 50% mortality was calculated using Table curve 2D v5.01 software. The test was performed in triplicate.

α-Amylase inhibition assay: Antidiabetic activity of test samples was assessed by α-amylase inhibition assay using standard protocol with minor modifications (Kim *et al.*, 2000). The reaction plate containing 25 µl α-amylase enzyme (0.14 U/ml), 15 µl of phosphate buffer (pH 6.8), 10 µl of test samples (4 mg/ml DMSO) and 40 µl of starch solution (2 mg/ml in potassium phosphate buffer) was kept at 50°C for 30 min in 96 well plate trailed by addition of 20 µl of 1 M HCl to halt the reaction. Afterwards 90 µl of iodine reagent (5 mM potassium iodide, 5 mM iodine) was transferred to each well and Acarbose (250 µM) served as positive control. Negative control was prepared without plant extracts whereas blank was prepared without extracts and amylase enzyme; each being substituted by equal quantities of buffer. Absorbance of reaction mixture was recorded at 540 nm.

Inhibitory activity was expressed as % α-amylase inhibition/mg of extract and calculated as follows:

$$\% \alpha\text{-amylase inhibition} = (\text{As} - \text{An}) / (\text{Ab} - \text{An}) \times 100$$

where An = Absorbance of negative control, As = Absorbance of sample and Ab = Absorbance of blank well.

In vivo studies

Experimental animals: Sprague-Dawley albino, 5-10 months old rats of both male and female sex (250-300 g) were used to study the antidiabetic activity. Animals were housed under standard laboratory conditions (temperature $22 \pm 2^\circ\text{C}$ and $45 \pm 5\%$ relative humidity with 12 hour day/night cycle at the animal house of National Institute of Health, Islamabad, Pakistan. All animals received standard laboratory pellet diet and water ad libitum. The study was approved by the Bioethical committee of Quaid-i-Azam University Islamabad (protocol # BEC-FBS-QAU-04).

Induction of alloxan induced diabetes: Previously refrigerated (4°C) alloxan monohydrate was dissolved in normal saline at room temperature and injected intraperitoneally at a dose of 120 mg/kg to the overnight fasted rats (Ojiako *et al.*, 2016). In order to prevent alloxan induced severe hypoglycaemia, rats were supplemented with 20% glucose solution for the first 6 hours and were then kept with free access to 5% glucose solution for the next 24 hours. After 72 hours of alloxan injection, the rats with fasting blood glucose level > 250 mg/dl were considered hyperglycemic and were selected for the *In vivo* studies. The animals were randomly divided into 9 groups (n = 6) and were designated on the basis of treatments received at regular intervals of 7 days for 21 days as follows:

- Group A; Control, normal healthy rats.
- Group B; Untreated diabetic rats.
- Group C; Diabetic rats were given standard glibenclamide (0.6 mg/kg, p.o.).
- Groups D, E and F; Diabetic rats received 300 mg/kg, p.o. of aqueous, ethanol and chloroform extracts of BO flowering heads, respectively.
- Groups G, H and I; Diabetic rats received 300 mg/kg, p.o. of aqueous, ethanol and chloroform extracts of CA seeds, respectively.

Extracts selected from *In vitro* studies were administered by the oral gavage to rats. Aqueous and ethanolic extracts were dissolved in normal saline (extract vehicle) whereas, <1% DMSO was used as extract vehicle in case of glibenclamide and chloroform extract. The solutions were freshly prepared before oral administration on each day. The body weight and fasting blood glucose level were checked at day 0, 7, 14 and 21 by digital weighing balance (Ohaus corporation PA214C, USA) and On-call plus ® glucometer (Acon lab, USA) respectively. At the end of the study all the animals were euthanized with excess (120 mg/kg) of sodium pentobarbital IV injection.

Biochemical analysis: At day 22, all the rats were deeply anesthetized with chloroform and blood from the horizontal axis of the sternum was collected in vacuumed blood collection tubes (plain Red, BD Vacutainer, USA) (Haq *et al.*, 2012). The separated serum was stored in Eppendorf tubes at -20°C for further analysis of biochemical parameters. For the determination of fasting plasma glucose, total cholesterol, alanine transaminase (ALT) and serum urea levels, the samples were analysed using a semi-automated biochemical analyser (Tecno 786 audit diagnostic, Ireland) using commercial kits according to manufact.

(a). Fasting plasma glucose level (FPGL): Enzymatic glucose oxidase colorimetric method using commercially available kit (Live diagnostic Inc, Canada) was employed for FPGL determination (Ojiako *et al.*, 2016).

(b). Total cholesterol (TC): Commercially available kit (Live diagnostic Inc, Canada) containing reagent (cholesterol oxidase, cholesterol esterase, peroxidase, 4-aminoantipyrine, phenol and buffer) was employed for the estimation of TC (Atawodi *et al.*, 2014).

(c). Alanine transaminase (ALT): For the photometric determination of serum ALT level, commercial kit (ALT Activity Assay Kit MAK055 Merck, France) was used (Luan & Sun, 2015).

(d). Serum urea: Commercially available kit (Live diagnostic Inc, Canada) for the estimation of serum urea level was used (Luan & Sun, 2015).

Statistical analysis

All *In vitro* assays were performed in triplicate; mean and standard deviation were calculated. For LC₅₀ and IC₅₀ Table curve 2D v5.01 software was used. One way analysis of variance (ANOVA) followed by least significant difference (LSD) test was applied for antioxidant assays. For *In vivo* testing, One way analysis of variance (ANOVA) followed by LSD test was applied for ALT, total cholesterol and serum urea estimation studies. Two way ANOVA was applied for blood glucose level and body weight (where n = 6) determination studies. For ANOVA, SPSS (SPSS for Windows, V16.0. Chicago, SPSS Inc., Chicago, IL) were used. P value of <0.05 was used to assign level of significance.

Results and Discussion

Extract recovery: The percentage of BO and CA extracts recovered from solvents of escalating polarity is summarized in Fig. 1. Highest extraction efficiency in terms of extract yield was observed in the aqueous extract of CA and BO i.e. 10.2% and 9.0% respectively followed by ethanol extracts. For both the plants, lowest extract yield was obtained when pet-ether (BO = 1.83% and CA = 1.63%) was used as the extraction solvent. Selection of an appropriate extraction procedure is imperative for the standardization of herbal preparations as it results in the amputation of necessary soluble constituents, is a critical parameter for upscaling the bench scale to pilot plant level (Dhanani *et al.*, 2013)

and is usually different for different plant matrices (Złotek *et al.*, 2016). Sonication aided maceration, the extraction technique employed in this study utilizes high frequency and high intensity sound waves and solvents to recover desirable compounds from plant matrices. Chemical and physical characteristics of the materials are transformed due to the interaction and dissemination of ultrasound waves disrupting the cell walls, thereby, augmenting solvent's mass transport across the plant cells (Dhanani *et al.*, 2013). The ultimate objective of the present study was to evaluate the efficacy of four solvent systems: water, ethanol, chloroform and pet-ether in terms of the influence of their polarity on the extractability of bioactive molecules possessing particular effectiveness in diabetes therapeutics. A previous study to optimize extraction parameters for *Brassica oleracea* L. (cauliflower) demonstrated a significant difference in the extractability of each type of solvent employed and it was observed that aqueous solvent was most proficient across a number of parameters including extract yield, phenolic content as well as antioxidant activity which was in agreement with the results of our present study (Anwar *et al.*, 2013).

Phytochemical analysis

TPC and TFC: Polyphenols play a significant role in the management of diabetes as they modify lipid and carbohydrate metabolism, dyslipidemia, reduce hyperglycemia, and insulin resistance, reduce oxidative stress, improve adipose tissue metabolism and stress sensitive signaling pathways as well as other inflammatory conditions (Atawodiet *et al.*, 2014). They can also deter the development of long term complications of diabetes including nephropathy, cardiovascular disease, neuropathy, and retinopathy (Ojiako *et al.*, 2016). Flavonoids improve, stabilize and sustain the insulin secretion, pancreatic cells and human islets respectively (Ghasemi Pirbalouti *et al.*, 2014). They are among the listed several antidiabetic compounds, which exercise their hypoglycaemic properties via extra pancreatic mechanism of α -glucosidase modulation (Algariri *et al.*, 2013), α -amylase inhibition and have unveiled glycaemic control in streptozocin-induced rat model of type I DM (Ojiako *et al.*, 2015). Therefore, TPC and TFC was quantified in various solvent soluble extracts of BO and CA in order to extrapolate their antidiabetic prospective.

In the present study, a comparative analysis of the phenolic and flavonoid profile shows that CA extracts has a higher content of these phytochemicals than BO (Table 1). The total gallic acid equivalent phenols ranged from 184 ± 0.1 - 80.7 ± 1.1 μ g GAE/mg E for BO extracts and 406 ± 0.1 - 80.7 ± 2.2 μ g GAE/mg E in case of CA extracts. Highest amount of total phenolic content was quantified in the ethanol extracts of the subject plants whereas it was lowest in their pet-ether extracts. Phenolic content of BO in descending order is as follows; BO ethanol > BO aqueous > BO chloroform > BO pet-ether while the phenolic content of CA in descending order is CA ethanol > CA chloroform > CA aqueous > CA Pet-ether. The highest TFC in terms of quercetin equivalent was quantified in the ethanol extract of CA (361.9 ± 0.1 μ g QE/mg of E) followed by CA chloroform > BO ethanol > BO aqueous > BO chloroform > CA

aqueous > BO pet-ether > CA Pet-ether. A significant amount of phenols and flavonoids were present in almost all the extracts of BO and CA except for the pet-ether extract. Utilization of polar solvents enable extraction of significant amounts of phenolics and flavonoids and are frequently used for their recovery from plant matrices (Fatima *et al.*, 2015). Although the extraction efficiency of ethanol in terms of retrieval of TPC and TFC per mg of extract is higher than aqueous extracts; however, the extraction efficiency of aqueous extracts in terms of extract yield is almost double than the ethanol extracts. These findings suggest the oral intake of BO and CA plants with water as a valuable source of antioxidant and antihyperglycemic compounds. A positive correlation ($R^2 = 0.99$ for BO and 0.91 for CA) was observed among the phenolic and flavonoid contents of BO and CA therefore, the antioxidant potential of phenols might be attributed to the presence of flavonoids. BO is considered to be a phenolic rich crop (Wu *et al.*, 2004). Previously a phenolic content of 108 ± 6 mg GAE/100 g fresh weight was quantified in the methanol extract of BO using ultrasound aided maceration as the extraction technique (Parente *et al.*, 2013). The slight differences in its phenolic contents as reported in the aforementioned study might be endorsed to variation in other parameters such as extraction method, plant variety or solvent used. To the best of our knowledge this is the first report that have elucidated the phenolic and flavonoid profile of CA.

Biological evaluation

In vitro assays

FRSA, TRP and TAC: Multimode free radical scavenging, reducing power and total antioxidant capacity assays were performed to evaluate the antioxidant potency of the samples, the results of which are presented in Table 1. Maximum DPPH free radical scavenging efficiency of 92.70 ± 0.6 ($IC_{50} = 10.5 \pm 1.7$ μ g/ml) and $90.10 \pm 0.1\%$ ($IC_{50} = 8.20 \pm 2.3$ μ g/ml) was exhibited by the ethanol extracts of BO and

CA respectively while the least quenching action was manifested by their nonpolar extracts. The polar extracts of both plants i.e. BO ethanol (427.9 ± 0.1 μ g AAE/mg E) and CA aqueous (480 ± 2.1 μ g AAE/mg E) displayed maximum reducing power potential which decreased in the following order: CA aqueous > BO ethanol > BO aqueous > CA ethanol > CA chloroform > BO chloroform > BO pet-ether > CA Pet-ether. In case of ascorbic acid equivalent total antioxidant capacity indices, the ethanol extract of BO (223.7 ± 0.2 μ g AAE/mg E) and CA (257.2 ± 0.3 μ g AAE/mg E) was most agile for the retrieval of phytoconstituents with antioxidant capabilities whereas the most nonpolar pet-ether extract recovered least antioxidant metabolites. Diabetic complications associated with increased oxidative stress can be suppressed by antioxidants (Bhattacharya *et al.*, 2013). Generation of free radicals is preliminary step in the progression of numerous chronic disorders including DM (Vessal *et al.*, 2003). Generally antioxidants represent molecules responsible for the decline of stress and inhibition of chain oxidative reactions associated with free radicals by means of quenching of singlet oxygen and free radical scavenging or by complexing with pro-oxidant metals (Valko *et al.*, 2006). Various explorations have confirmed the protective impact of antioxidants against diabetes induced oxidative stress (Atawodi *et al.*, 2014) that explains our current interest in determining antioxidant potency of the subject plants by using multimode assays. A noteworthy free radical scavenging efficiency was exhibited by aqueous and ethanol BO extracts as compared to chloroform and pet-ether. The current scavenging results reinforce previous findings that most of the components having radical scavenging activity are soluble in polar solvents (Lenzen, 2008). In CA, among all the tested samples, the aqueous extract exhibited most prominent reducing power potential. The reducing properties are generally allied with the presence of reductones which have been linked to the antioxidant action through breakage of the free radical chain by donating a hydrogen atom. The multimode antioxidant potential of CA is reported for the first time in this study.

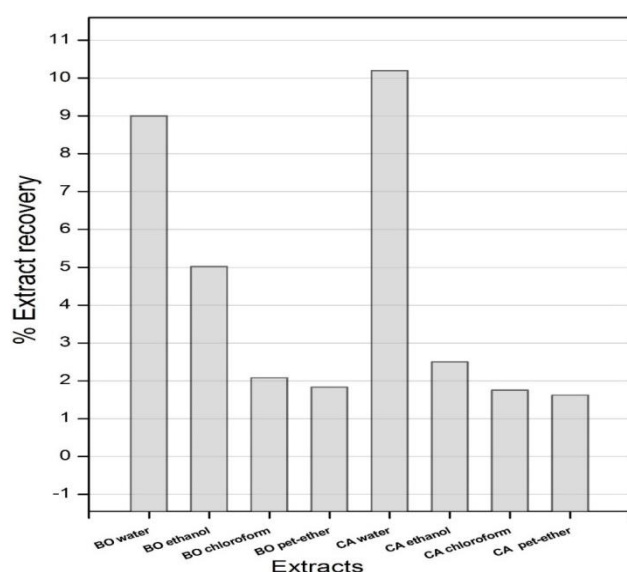


Fig. 1. Extract recovery (%) of *Brassica oleracea* var. *italica* (BO) flowering heads and *Cassia absus* (CA) seeds in distilled water, ethanol, chloroform and pet-ether solvents.

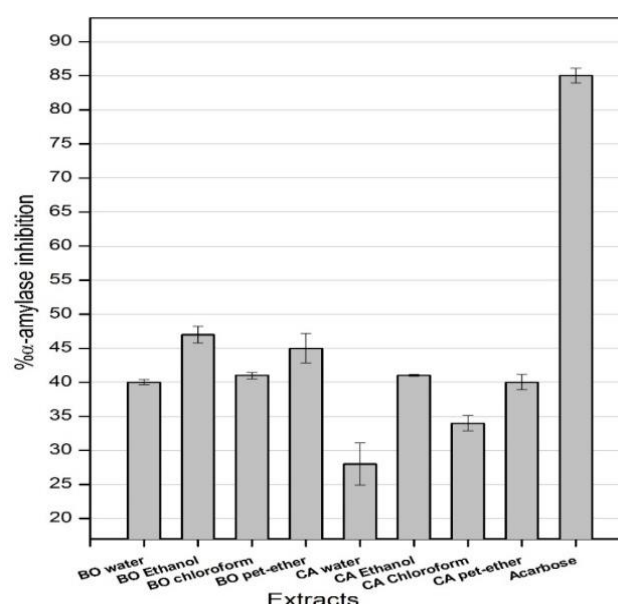


Fig. 2. Alpha amylase inhibition activity of *Brassica oleracea* var. *italica* (BO) and *Cassia absus* (CA).

Table 1. TPC, TFC, FRSA, TAC, TRP and α -amylase inhibition potential of aqueous, ethanol, chloroform and pet-ether extracts of *Brassica oleracea* var. *italica* (BO) and *Cassia absus* (CA).

Samples	TPC	TFC	FRSA	IC ₅₀	TAC	TRP	α -Amylase assay
	$\mu\text{g GAE/mg E}$	$\mu\text{g QE/mg E}$	% FRSA	$\mu\text{g/ml}$	$\mu\text{g AAE/mg E}$	$\mu\text{g AAE/mg E}$	% Inhibition
BO water	161 \pm 1.2	139.7 \pm 0.2	81.50 \pm 0.8 *	11.9 \pm 3.2	109.1 \pm 1.2 #	374.3 \pm 0.2#	40 \pm 0.4
BO ethanol	184 \pm 0.1	160.9 \pm 0.1 *	92.70 \pm 0.6 *	10.5 \pm 1.7	223.7 \pm 0.2*	427.9 \pm 0.1#	47 \pm 1.3*
BO chloroform	146.4 \pm 0.1	133.8 \pm 0.1	48.40 \pm 0.6	-	87 \pm 0.2	267.4 \pm 0.1	41 \pm 0.1
BO pet-ether	80.7 \pm 1.1	72.8 \pm 1.1	47.0 \pm 1.4	-	40.3 \pm 1.3	200 \pm 1.7	45 \pm 2.2*
CA water	207 \pm 0.1	89.5 \pm 0.3	75.00 \pm 0.1#	8.26 \pm 1.2	189.6 \pm 0.1	480 \pm 2.1*	28 \pm 3.1
CA ethanol	406.7 \pm 0.1	361.9 \pm 0.1 *	90.10 \pm 0.1 *	8.2 \pm 2.3	257.2 \pm 3.3 *	371.7 \pm 0.1 #	41 \pm 0.1
CA chloroform	351.5 \pm 0.6	214.7 \pm 0.9*	42.30 \pm 1.3	-	181.6 \pm 0.9	339.7 \pm 1.9 #	34 \pm 1.1
CA pet-ether	80.7 \pm 2.2	21.7 \pm 1.0	47.90 \pm 0.6	-	72.1 \pm 1.1	198.3 \pm 0.9	40 \pm 1.1
Acarbose	-	-	-	-	-	-	80.3 \pm 1.1

Extracts with statistically significant results are indicated by a superscript. In all of the results* indicate ($p < 0.01$) and # indicate ($p < 0.05$). Acarbose IC₅₀ = 33.73 \pm 0.12 $\mu\text{g/ml}$

α -Amylase inhibition potential: Control of postprandial hyperglycaemia is a practical approach for the management of diabetes and could be achieved through inhibition of enzymes that regulate carbohydrate hydrolysis such as α -glucosidase and α -amylase (Thenmozhi *et al.*, 2015). The contemporary carbohydrate hydrolysing enzymes such as miglitol, metformin and voglibose are associated with a number of adverse effects such as diarrhoea, flatulence and abdominal distention (You *et al.*, 2012). Therefore, quest for benign and operative inhibitors from natural sources is of paramount importance. The α -amylase inhibitory potential of BO and CA extracts is shown in Fig. 2. *In vitro* studies demonstrated that all the crude BO and CA extracts screened for this activity possess varied α -amylase inhibitory potential. BO extracts displayed greater enzyme inhibition as compared to CA. Among all the extracts, BO ethanol showed the highest activity (47 \pm 1.25%) in comparison to Acarbose (80.34 \pm 1.12% of α -amylase enzyme's activity with an IC₅₀ of 33.73 \pm 0.12 $\mu\text{g/ml}$). The order of decreasing activity in terms of percentage of amylase inhibition is as follows: BO ethanol > BO pet-ether > BO chloroform = CA ethanol > BO aqueous = CA pet-ether > CA chloroform > CA aqueous.

Brine shrimp cytotoxicity assay: For both the plants, the degree of lethality was directly correlated with the concentration of the extracts (Fig. 3). Highest mortality rate was at a concentration of 500 $\mu\text{g/ml}$ whereas lowest mortalities were observed at a concentration of 62.5 $\mu\text{g/ml}$. Only CA and BO pet-ether extracts (LC₅₀ 86.6 \pm 3.1 and 201.0 \pm 3.0 $\mu\text{g/ml}$ respectively) exhibited significant toxicity comparable to doxorubicin. The brine shrimp lethality test is acknowledged as a suitable procedure for principal evaluation of toxicity. It is an inexpensive and rapid method to find the bioactivity and correlate well with cytotoxicity of tested drugs (Fatima *et al.*, 2015). The cytotoxicity results of our present investigation inferred all the extracts to be sufficiently safe to proceed further for the animal studies except for the pet-ether extract. The toxicological aspects of CA are established for the first time in our present investigation.

In vivo evaluation

For both the plants, test extracts having low extract yield, less significant antioxidant potential, non-substantial α -amylase inhibitory activity and a comparatively higher cytotoxicity were excluded from animal studies. On the basis of aforementioned criteria, pet-ether extracts of both the plants were excluded from the animal studies.

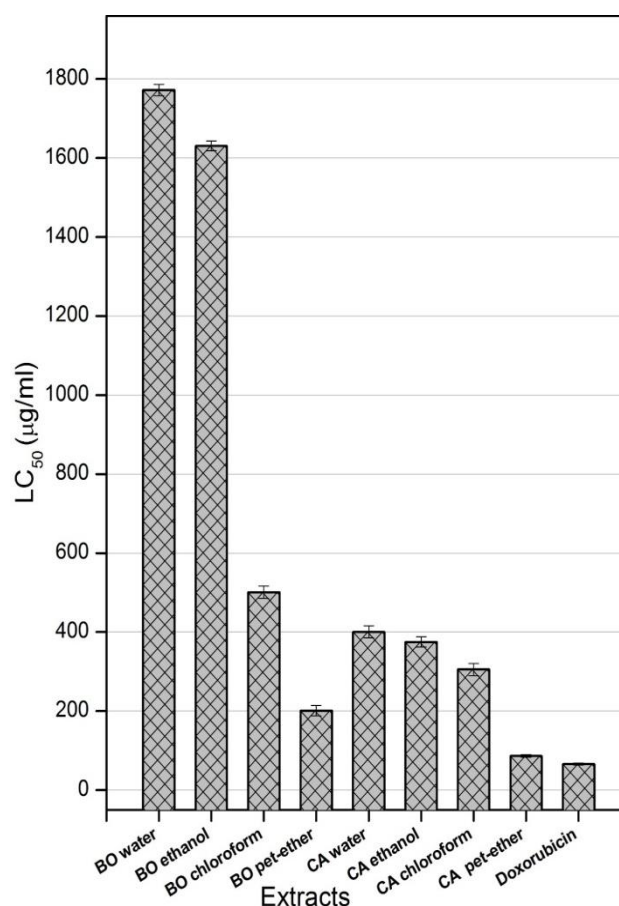


Fig. 3. Median lethal concentration (LC₅₀) of *Brassica oleracea* var. *italica* (BO) and *Cassia absus* (CA) extracts against brine shrimp nauplii.

Table 2. Effect of *Brassica oleracea* var. *italica* (BO) and *Cassia absus* (CA) extracts on fasting blood glucose level and body weight in alloxan induced diabetic rats.

Groups	Parameters	Day 0	Day 7	Day 14	Day 21
Group A (Normal Control)	Blood glucose (mg/dl)	99.00 ± 2.0	95.7 ± 2.6*	95.5 ± 1.4*	94.2 ± 3.0*
	Body weight (g)	282.30 ± 6.5 [#]	294.50 ± 3.9	302.8 ± 3.9	305 ± 4.2 [#]
Group B (Diabetic Control)	Blood glucose (mg/dl)	366.70 ± 9.5	358.7 ± 6.84	316.0 ± 3.1 [#]	297.2 ± 4.8 [#]
	Body weight (g)	285.3 ± 5.2 [#]	273.00 ± 4.3	270.2 ± 5.3*	270.2 ± 5.3*
Group C (Glibenclamide)	Blood glucose (mg/dl)	356.0 ± 3.3	161.3 ± 7.5*	156.0 ± 3.9*	153 ± 9.4*
	Body weight (g)	279.2 ± 3.2 [#]	279.2 ± 3.2 [#]	293.3 ± 4.4 [#]	297.2 ± 4.0
Group D (BO Water treated)	Blood glucose (mg/dl)	286.2 ± 3.5	173.5 ± 6.1*	164 ± 4.3*	161.5 ± 4.4*
	Body weight (g)	285.0 ± 1.3 [#]	285.0 ± 3.2 [#]	287.2 ± 1.4 [#]	290.8 ± 2.1
Group E (BO Ethanol treated)	Blood glucose (mg/dl)	348 ± 5.4	238 ± 9.4 [§]	237.2 ± 3.2 [§]	223.3 ± 10.3 [§]
	Body weight (g)	274.8 ± 6.3 [#]	274.8 ± 5.2	284.8 ± 2.2	294.3 ± 2.2
Group F (BO Chloroform treated)	Blood glucose (mg/dl)	293 ± 9.6	281 ± 6.8 [§]	271.8 ± 7.4 [§]	251.5 ± 8.7 [§]
	Body weight (g)	283.0 ± 3.5 [#]	283 ± 4.5 [#]	283.8 ± 3.8	285.7 ± 8.8
Group G (CA Water treated)	Blood glucose (mg/dl)	374.7 ± 8.2	186.7 ± 5.6*	164 ± 5.7*	142.3 ± 7.1*
	Body weight (g)	278.5 ± 4.2 [#]	296.8 ± 3.0	305.3 ± 3.9*	305.3 ± 3.0*
Group H (CA Ethanol treated)	Blood glucose (mg/dl)	299.8 ± 7.9	219.7 ± 4.6 [§]	195.2 ± 5.8 [§]	186.2 ± 8.7 [#]
	Body weight (g)	281.0 ± 3.8 [#]	300.3 ± 3.1 [#]	292.3 ± 2.2	292.3 ± 4.0 [#]
Group I (CA Chloroform treated)	Blood glucose (mg/dl)	350.80 ± 9.7	188.8 ± 16.8 [#]	169.5 ± 6.1 [#]	158 ± 7.4*
	Body weight (g)	281.0 ± 4.2 [#]	295.7 ± 4.4	298.7 ± 2.7 [#]	298.7 ± 2.7

Two way ANOVA along with multiple comparison test was applied. All data are expressed as mean ± SE (n = 6). Where * < 0.001 compared to normal control; * < 0.001 compared to diabetic control. [#] indicated that there is non-significant difference between the groups. Similarly for blood glucose level, multiple comparison test of normal control and different extracts of BO and CA (300 mg/kg) at different time intervals were applied where * indicates p<0.05, * p<0.01, [#] p<0.001, [§] P = .000

Effect on body weight and blood glucose level: In order to induce diabetes intraperitoneally, a cytotoxic drug alloxan monohydrate was used as reported in many studies (Saadia *et al.*, 2005). Weight loss is one of the main symptoms of diabetes (Snipelisky & Ziajka, 2012). After alloxan treatment almost all the rats showed significant weight loss due to lipolysis and gluconeogenesis from muscle proteins (Ojiako *et al.*, 2015). The effect of daily, continuous oral treatment with BO and CA extract on body weight and fasting blood glucose level of alloxan induced diabetic and normal control rats is shown in Table 2 and Figs. 4 and 5. The weight of diabetic control rats (270.2 ± 5.3 g) was reduced after 21 days; however, there was an increase in the body weight of the extract treated groups (285.7 ± 8.8-305.3 ± 3.0 g). At day 0, all the groups exhibited non-significant difference in the body weight (p>0.05) except animals administered with BO ethanol extract (p<0.05). At day 7, non-significant difference in body weight (p>0.05) between treatment groups (274.8 ± 5.2-300.3 ± 3.1 g) and normal control (294.50 ± 3.9 g) was observed except diabetic control (273.00 ± 4.3 g). Same trend was followed at day 14 i.e. alloxan induced diabetic control (270.2 ± 5.3 g) exhibited significant weight loss as compared to treatment groups (283.8 ± 3.8-298.7 ± 2.7 g) indicating that all the extracts showed substantial protection against weight loss. In comparison to diabetic control (270.2 ± 5.3 g), BO extract treated groups (283.8 ± 3.8-287.2 ± 1.4 g) exhibited less protection against weight loss as compared to CA extract treated groups (298.7 ± 2.7-305 ± 3.9 g). At day 21, diabetic rats (270 ± 5.3 g) demonstrated consistent weight loss as compared to normal control (305 ± 4.2 g). After 21 days of treatment, all the extract treated groups demonstrated significant protection against weight loss as compared to glibenclamide treated rats (297.2 ± 4.0 g) as follows; CA aqueous (305.3 ± 3.0 g) > CA chloroform > BO ethanol > CA ethanol > BO aqueous > BO chloroform (285.7 ± 8.8 g). Results of fasting blood glucose level are

shown in Table 2 and Fig. 5. At day 0, the blood glucose levels of alloxan treated groups (366.70 ± 9.5 mg/dl) were significantly (p<0.05) higher than normal control (99.00 ± 2.0 mg/dl). At day 7, the blood glucose levels of extract treated groups were significantly higher than normal control (95.7 ± 2.6 mg/dl) but less than diabetic control (Diabetic control; 358.7 ± 6.84, treated groups; 173.5 ± 6.1-281 ± 6.8 mg/dl). When equated with glibenclamide (161.3 ± 7.5 mg/dl), CA water (186.7 ± 5.6 mg/dl), CA ethanol (219.7 ± 4.6 mg/dl) and BO water (173.5 ± 6.1 mg/dl) extracts exhibited comparable activity. At day 14, all treatment groups showed comparable results with glibenclamide except BO ethanol which although showed protection against diabetes but to lesser extent than other treatments. At day 21, same trend was followed in all the extract treated animals i.e. they showed significant protection against alloxan induced hyperglycaemia (p<0.05) except BO ethanol which had some protective effect but not comparable to glibenclamide (p<0.05). The decrease in blood glucose level by treatment in all the groups in descending order (at day 21) is as follows: CA aqueous (142.3 ± 7.3 mg/dl) > CA chloroform > BO aqueous > CA ethanol > BO ethanol > BO chloroform (251.5 ± 8.7 mg/dl). All the animals treated with extracts gained weight during the study. Treatment with both CA and BO extracts presented significant antihyperglycaemic action at varying extents. Some of the mechanisms of hypoglycaemia as reported by earlier studies include; restoration of the function of damaged pancreas, mimicry of insulin action, alteration in the activity of hepatic enzymes involved in glucose metabolism and decreased intestinal absorption of glucose (Grover *et al.*, 2002). The hypoglycaemic and antioxidant properties of extracts may protect pancreas from deleterious effects of prolonged high glucose level. It was observed that CA aqueous extract (group G) was most proficient in lowering the alloxan induced hyperglycaemia as compared to standard control

(group C) but could not sufficiently inhibit amylase enzyme therefore, the *In vivo* antidiabetic potential of CA aqueous extract might be extrapolated to the presence of a higher content of phenols, flavonoids, reductones and other antioxidant moieties as quantified in our present exploration. Likewise, among all the BO extracts, BO ethanol demonstrated a noteworthy *In vitro* antioxidant potential but did not perform well *In vivo* antidiabetic study. Exact mechanisms of action is yet to be determined.

Effect on total cholesterol, ALT and serum urea:

According to one way ANOVA and LSD test all of the treatment groups have significant difference ($p < 0.05$) among them for total cholesterol, ALT and serum urea levels (Table 3). At the end of the study, ALT levels of alloxan-induced diabetic rats (Group B) was significantly higher (155.8 ± 28.7 U/l) compared to normal control rats (40.3 ± 4.1 U/l). Whereas, the daily oral administration of glibenclamide (0.6 mg/kg) for 21 days significantly decreased (53.3 ± 1.7 U/l) the ALT levels in diabetic rats. Treatment of diabetic rats with BO aqueous (70.3 ± 5.3 U/l), BO chloroform (73.4 ± 20.5 U/l) and CA aqueous extract (69.5 ± 5.8 U/l) for 21 days resulted in a significant decline in ALT level compared to diabetic control groups.

In case of total cholesterol, only BO aqueous extract (group D; 136.2 ± 6.3 mg/dl) and glibenclamide (group C; 129 ± 2.1 mg/dl) presented statistically significant results

($p < 0.05$) as compared to normal control (group A; 102.8 ± 10.9 mg/dl). CA water (group G; 142.3 ± 8.0 mg/dl) and BO chloroform (group F; 160 ± 12.5 mg/dl) extracts also demonstrated comparable reduction in diabetes induced hypocholesteremia as compared to standard control group C. In case of serum urea levels, almost all the values were within normal range and only the CA chloroform extract (19.2 ± 1.2 mg/dl) exhibited statistically significant difference ($p < 0.05$) as compared to normal control.

A significant increase in total cholesterol and ALT levels was observed in untreated diabetic controls due to alloxan induced diabetes which is consistent with previous reports (Hamden *et al.*, 2009). Increase in ALT levels due to cellular damage are an indication of liver injury (Kim *et al.*, 2008). Blood urea nitrogen is basically a breakdown product of proteins and is used as an indicator of impaired renal function (Beier *et al.*, 2011). Elevated total cholesterol level in alloxan induced diabetes indicate impaired lipid metabolism (Nahar *et al.*, 2010). All of the extract treated groups seemed to improve the alloxan induced elevated levels of the aforementioned biochemical parameters indicating their possible role in the management of diabetic complications. According to some previous studies BO has the ability to boost liver enzymes for detoxification (Aspry & Bjeldanes, 1983; Guerrero-Beltran *et al.*, 2012). No such previous study about the effect of CA extracts on altered biochemical parameters is available.

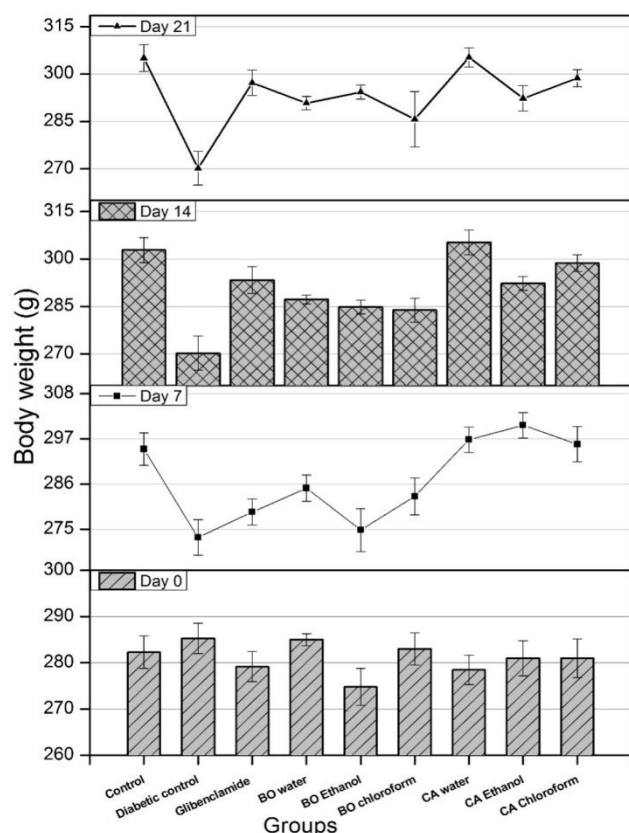


Fig. 4. Effect of treatment of aqueous, ethanolic and chloroform extracts of *Brassica oleracea* var. *italica* (BA) and *Cassia absus* (CA) on body weight of Alloxan induced diabetic rats determined on day 0, 7, 14 and 21 of treatment. Diabetic treatment groups were given (Glibenclamide at 0.6 mg/kg p.o (group C) and plants extracts at a dose of 300 mg/kg p.o. (Group D-I). Values are presented as mean \pm SD ($n = 6$).

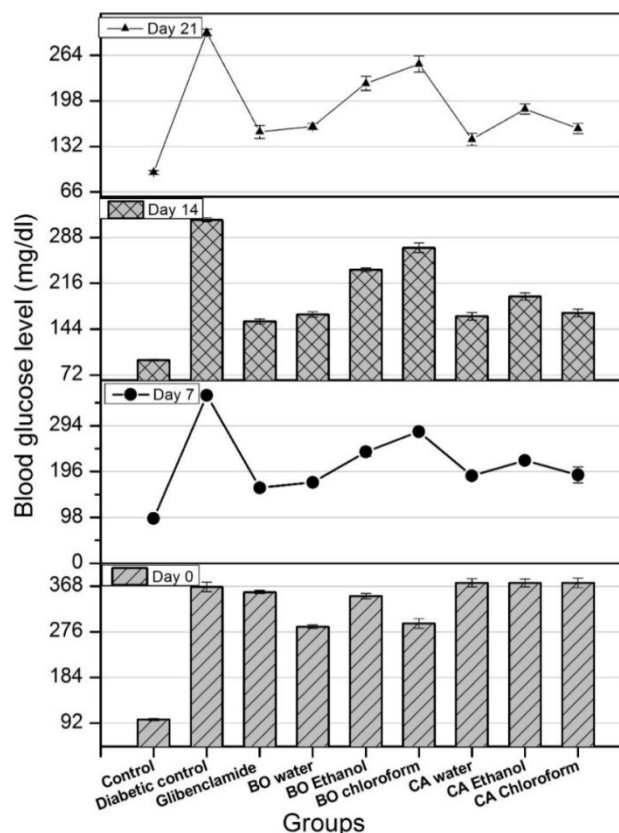


Fig. 5. Effect of treatment of aqueous, ethanolic and chloroform extracts of *Brassica oleracea* var. *italica* (BA) and *Cassia absus* (CA) on blood glucose level of alloxan induced diabetic rats determined on day 0, 7, 14 and 21 of treatment. Diabetic treatment groups were given Glibenclamide at 0.6 mg/kg p.o (group C) and plants extracts at a dose of 300 mg/kg p.o. (Groups D-I). Values are presented as mean \pm SD ($n = 6$).

Table 3. The impact of *Brassica oleracea* var. *italica* (BO) and *Cassia absus* (CA) on total cholesterol, ALT and blood urea levels in alloxan induced diabetic rats.

Groups	Total cholesterol mg/dl	ALT U/l	Urea mg/dl
Normal control (A)	102.8 ± 10.9*	40.3 ± 4.1*	11 ± 0.9
Diabetic control (B)	200.8 ± 24.5 [#]	155.8 ± 28.7 [#]	5.8 ± 0.3
Glibenclamide (C)	129 ± 2.1*	53.3 ± 1.7*	16.6 ± 2.6
BO water (D)	136.2 ± 6.3*	70.3 ± 5.3*	6.9 ± 0.3
BO ethanol (E)	162.7 ± 10.1 [#]	119.5 ± 18.1 [#]	11.6 ± 1.4
BO chloroform (F)	160 ± 12.5*	73.4 ± 20.5*	15 ± 1.6
CA water (G)	142.3 ± 8.0*	69.5 ± 5.8*	14.5 ± 1.0
CA ethanol (H)	181.2 ± 8.1 [#]	133.8 ± 17.7 [#]	16.5 ± 1.8
CA chloroform (I)	186.2 ± 4.5 [#]	119.5 ± 17.6	19.2 ± 1.2*

Effect of treatment on biochemical parameters in alloxan-induced diabetic and normal control rats (Glibenclamide at 0.6mg/kg and BO and CA extracts at a dose of 300 mg/kg p.o.). Each value represent mean ± SD, n = 6. * p<0.05, * p<0.01, [#] p<0.001.

Conclusions

The results of *In vitro* antioxidant, α -amylase inhibition and *In vivo* antidiabetic assays extrapolate the traditional use of these folklores as anti hyperglycaemic agents. The present study proposes that the aqueous and chloroform extracts of CA, while aqueous extract of BO as an expedient source of antidiabetic compounds. Likewise, the results of our multimode antioxidant assays suggest that these herbal cocktails may offer additional unique benefits for the management of diabetic complications such as hepatorenal and pancreatic protection from chronic disease. The present study calls for further research aimed at isolating the bioactive compounds responsible for the observed activity that could serve as novel gallows in quest for diabetes management.

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