Research article

Antioxidant and antidiabetic activities of *Holarrhena antidysenterica*

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ABSTRACT

Introduction and Aim: *Holarrhena antidysenterica* (Kutaja) belonging to the Apocynaceae family is widely grown in India and used in traditional systems of medicine. Presence of polyphenols, flavonoids and alkaloids make the plant useful in treating metabolic disorders. This study presents comparison of antioxidant property between leaf and bark of *H. antidysenterica* followed by *in vivo* experimental validation of anti-diabetic properties of leaf extracted in methanol.

Materials and Methods: Bark and leaf of *H. antidysenterica* extracted in methanol by Soxhlet apparatus and quantified the phytochemicals present with respective standards. Antioxidant properties was estimated by DPPH scavenging assay. High Fat Diet C57BL/6 mice model was established and treated with leaf extract of *H antidysenterica*. Levels of TNF alpha and blood glucose were monitored.

Results: The IC_{50} values of bark and leaf of *H. antidysenterica* were 10.15 and 8.56 µg/ml respectively on DPPH scavenging assay. The established Sandwich ELISA would detect TNF alpha with the detection limit of 0.011 µg/ml. This ELISA was used to estimate TNF alpha in the mice serum, and found that there was an increase in TNF alpha level in HFD animals (29.76 ± 5.1 µg/ml) which was lowered by the treatment of leaf extract (22.08 ± 2.08 µg/ml). The blood glucose levels were also reduced upon treatment with leaf extract from 130.83 ± 6.43 mg/dl to 111 ± 2.9 mg/dl.

Conclusion: Presence of high amount of phenolic compounds in leaf is responsible for its antioxidant property, whereas high amount of fat content in bark with moderate amount of phenolic compounds are the likely cause of its close-matched hydroxyl radical scavenging activity despite lesser content of phenolic compounds than leaf. Leaf also has anti-diabetic activity, having shown by reduction in TNF alpha and blood glucose levels by *in vivo* mice model. Phenols, flavonoids and tannins are the abundant phytochemicals found when leaves were extracted in methanol which might be responsible for its antidiabetic activity.

Keywords: *Holarrhena antidysenterica*; phytochemical analysis; antioxidant; TNF alpha; glucose uptake.

INTRODUCTION

Oxidative stress is a condition when the balance between oxidants and antioxidants is lost. Antioxidants are classified into two groups: enzymatic and nonenzymatic. Enzymatic antioxidants produced in our body include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), whereas glutathione, vitamin C, vitamin E, carotenoids, uric acid from fruits and vegetables are considered as nonenzymatic antioxidants (1).

High calory intake and impaired metabolism leads to inflammation in the visceral organs. This affects many vital organs involving inflammatory pathway through macrophages and TNF alpha. The accumulating free fatty acids and increased pro-inflammatory cytokines converge to form insulin resistance (2).

Many medicinal plants are the abundant sources of nonenzymatic antioxidants. It is well known that natural phenolic compounds present in plants confer the host antioxidant property through mechanisms like hydrogen atom transfer or single electron transfer (3). A comparative evaluation of four such aromatic plants in the Indian subcontinent showed correlation between total phenolic content of the plant parts and its DPPH scavenging activity (4).

Plants like *H. antidysenterica*, commonly known as Kutaj or Kurchi are used for treatment of many diseases like cancer (5), metabolic disorders like diabetes (6), atherosclerosis, and other cardiovascular diseases caused by oxidative stress (7). Its antioxidant activity can be detected

experimentally through *in vitro* and *in vivo* reactions. Polar solvents like methanol or ethanol dissolve most of the phytochemicals, methanolic extracts has lower genotoxicity on *in vivo* models with high radical scavenging activity (8).

In this study we present an experimental comparison of phytochemical content in bark and leaf and antioxidant property of the methanol extract. Testing of leaf on *in vivo* model for its antidiabetic activity was also carried out. To our knowledge, this is a unique study on antioxidants combining *in vivo* studies on reduction of TNF alpha by experimental validation.

MATERIALS AND METHODS

Origin, distribution and morphology

Apocynaceae family of plants are distributed in Asia, tropical areas of Africa, Madagascar, India, Philippines and Malayan Peninsula (9). *H. antidysenterica* grows as a large tree of 30 to 40 feet in height, with white flowers arranged in cluster (Fig. 1a). Petals are salver shaped overlapping towards right side. Fruits are long follicles, about half inch in size with a milky latex. Bark is greyish-brown and rough (Fig. 1b) whereas the stem is white and soft. Leaves are simple, large, arranged opposite to each other, oval shaped, papery, and smooth or hairy. Follicles have white warty spots on the surface. Dried fruits break open releasing numerous flat seeds with brown hairs (10).

Fig. 1: (a) Leaf and flowers, (b) Bark of Kurchi, *Holarrhena antidysenterica*

Collection of plant material

The plant samples of H. antidysenterica was collected from a village near Soraba taluk, Shivamogga district, Karnataka. The plant materials were identified and authenticated by Dr. K. Kotresha. Dept. of Taxonomy and Floristic laboratory, Karnataka Science Collage, Dharwad, Karnataka, India. vide letter no. 258. The herbarium is kept at Karnataka Science Collage, Dharwad with accession no. 19553 for further reference. Seasonal variation in secretion of phytochemicals was ruled out by mixing samples collected throughout the year. The plant material was shade-dried and powdered in the grinder before taking for further analysis (11).

Preparation of methanolic extract

Polar solvents like methanol or ethanol can dissolve alkaloids, flavonoids, tannins, carbohydrates, steroids, terpenoids and proteins, which are the major components of interest in plants (8). Since genotoxic activity of methanolic extract has been shown to be the lowest among the common polar solvents (9), it is reported to contain high radical scavenging activity (4). *H. antidysenterica* leaf and bark were subjected to extraction in methanol. Leaf and bark powder, 2 g each were extracted individually in Soxhlet apparatus in 200 mL of methanol (HiMedia, AS059). Extraction was carried out till the solvent in extraction chamber turned colorless. The semi-solid oleoresin was then concentrated by air drying and used for further analysis, which is henceforth referred as analyte.

Phytochemical analysis

Qualitative assessment of fat, carbohydrates, protein and amino acid, alkaloid, glycosides, saponins, tannins, phenolic content, flavonoids, terpenoids and steroids were done according to the protocols by Gollo *et al.,* (11), Kunatsa *et al.,* (12) and Tanvir *et al.,* (13). Detailed protocol is given in the supplementary document. Phytochemicals responsible for the antioxidant property were quantified in both leaf and bark extracts.

Estimation of carbohydrates

Total carbohydrates was determined using the protocol described by Gollo *et al.,* protocol (11). The green to dark green colour was read at 630 nm and plotted on standard curve of glucose to quantify carbohydrate.

Estimation of proteins and amino acids

The total protein and amino acid of both extracts was determined by Folin-Ciocalteu's colorimetric method as described earlier (11). Total protein and amino acid contents were expressed as mg of bovine serum albumin equivalents per g of the extract.

Estimation of alkaloids

The total alkaloid content of the extracts was determined using method described by Lokapur *et al.,* (11, 14). The alkaloid content was calculated in percentage.

Estimation of tannins

The total tannin content of the extracts was determined using Folin-Ciocalteu's colorimetric method (13). Total tannins were expressed as mg of tannic acid equivalents per g of the extract.

Estimation of phenolic compounds

The total phenolic compounds of both extracts were determined by Folin-Ciocalteu's colorimetric method as described earlier (11, 14). Total phenolic contents were expressed as mg of gallic acid equivalents per g of the extracts.

Estimation of flavonoids

The total flavonoid contents of both the extracts were determined using the aluminium chloride assay (11, 14). Total flavonoid contents were expressed as mg of quercetin equivalents per mg of the extract (QE)/g extracts.

Estimation of fat

Total fat content of both the extracts was determined by previously described method (15). The fat content was calculated in percentage.

Determination of antioxidant property

Free radical scavenging effect of plant extract was determined using the 2-diphenyl- 1picrylhydrazyl (DPPH) with slight medications of the method described by (14). Briefly, the concentrations (1-100 µg/ml) of extracts were prepared. DPPH solution (6x10-5M) was prepared in ethanol and 1 ml of this solution was mixed with the same volume of leaf and extracts and standard ascorbic acid solution separately. The mixture was kept for incubation at dark for 30 minutes in room temperature and the optical density was measured at 517 nm. The level of DPPH purple decolorization to DPPH yellow confirmed the scavenging efficiency of the extract. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. DPPH scavenging activity (%) = AC-AT/AC× 100 Where AC- is the absorbance of the control reaction (1 ml of ethanol with 1 ml of DPPH solution), AT - is the absorbance of the test sample. The results were analysed in duplicates. The IC_{50} value is the concentration of sample required to inhibit 50 % of the DPPH free radical.

Making polyclonal antibody

Two male New Zealand white rabbits of 4-6 weeks of ~ 2 kg were taken for the experiment. Ethical committee clearance no. Invivo/033 issued from Invivo Biosciences. On day 0, pre-immune blood was collected (PIS) and the rabbits were immunized at sub cutaneous region with 300 µg/rabbit of TNF alpha protein. After 21 days of immunization rabbits were given 1st booster with 150 µg/rabbit. 2nd booster again after 21 days with 300 µg/rabbit was administered. Hyper-immune sera (HIS) were collected on the 10th day after 2nd booster and the same schedule was followed for further boosters (16). Antibody titer of both the rabbits was checked by In-direct ELISA. Based on the titer results 1 ml serum from 2nd rabbit was taken for Protein A purification. The Protein A (Merck, GE17-5199-01) beads was equilibrated with 20 mM Sodium Phosphate buffer pH 7.4. After equilibrating Protein A beads were incubated with 1 ml of antisera mixed with 9 ml of equilibration buffer with gentle rocking at 4°C for overnight. The flow through was collected the next day in a fresh 15 ml tube. The column was washed with 20 ml of 20 mM Sodium Phosphate buffer pH 7.4. The bound IgG were eluted with 50 mM Glycine, pH 2.8 into tubes containing 1M Tris-HCl, pH 8.0 (17). Positive elutes fractions were dialyzed against 1X PBS, pH 7.4 after checking for the presence of protein by Coomassie blue stain. Purified antibody was quantified by spectrophotometer assay.

Making detection for the assay

Antibody 0.5 mg at 10 mg/ml concentration was taken for conjugation, buffer exchange was done by dialyzing it against sodium bicarbonate buffer pH 9.6. 0.5 mg of HRP (Invitrogen, 31490) was dissolved in 0.5 ml of 50 mM PBS pH 7.2 to get a concentration of 10 mg/ml. Freshly prepared Sodium meta periodate (Signa, S1878) solution 0.1 ml was added to 1 ml of enzyme solution to make the working concentration of 8 mM Sodium meta periodate concentration in the reaction mixture. The mixture was kept mixing for 20 min in dark. The color change can be noted from Brownish/gold to green as the reaction proceeds. After 20 min of incubation the oxidized HRP was purified by dialyzing with sodium bicarbonate buffer pH 9.6. The antibody solution was mixed with the enzyme solution at a ratio of 1:1 (V/V). The mixture was incubated for 2 hrs at room temperature in a cyclomixer in dark. At the end of 2 hrs 10 µl of 5 M Sodium Cyanoborohydride (Sigma, 156159) was added to 1 ml of reaction solution to stop the reaction. The solution was incubated for 30 min mixing in dark at room temperature. The unreacted aldehyde sites were blocked by the addition of 50 µl of 1M Ethanolamine (sigma, 411000), pH 9.6 per ml of conjugation solution. The solution was incubated for 30 min mixing in dark at room temperature. Conjugate was purified from excess reactants by dialyzing it against 50 mM Tris-HCl pH 8.0. Filter and sterilize the HRP-conjugate by passing it through 0.22 µ filter. The conjugate was mixed with 1:1 of stabilization buffer: Glycerol 30%, Sucrose 0.5%, BSA 1%, Magnesium chloride 0.01%, Tween- 20 0.01% (18).

ELISA assay establishment and validation

The assay was established and validated according to the guidelines(19, 20). Detailed protocol for the establishment of different assay parameters is given in the supplementary document. Standards of TNF alpha protein starting from 3 to 0.0012 µg/ml concentration

with 1.75 times dilutions was prepared in assay matrix containing C57BL/6 mice sera to check the matrix interference. Matrix components can potentially interfere with the antibody binding. Therefore, the potential for variable matrix-related interference was evaluated in ten independent sources of C57BL/6 mice serum(21). The above established assay was validated by performing the assay on three different days by three different people with triplicates.

Sandwich ELISA protocol

Capture antibody was coated at 0.5 µg/ml in sodium-bi-carbonate buffer (pH 9.4) on to the ELISA plats, incubated overnight at 4ºC. After overnight incubation the content of the wells were discarded, washed with PBS and blocked with blocking buffer (1 % skimmed milk in PBST) (Himedia, GRM1254) for 1 hour at room temperature. After 1 hour of blocking the content of the wells were discarded, and analyte was added prepared in blocking/assay buffer, the plates were incubated for 1 hour at room temperature. After incubation the plates were washed three times with 1 X Phosphate Buffered Saline with 0.05 % Tween-20 (pH 7.2) followed by three times with 1 X Phosphate Buffered Saline (pH 7.2) and detection antibody at 1:10K was loaded to the wells in blocking buffer. The plate was incubated for 1 hour at room temperature. After 1 hour incubation, the plat was washed three times with 1 X Phosphate Buffered Saline with 0.05 % Tween-20 (pH 7.2) followed by three times with 1 X Phosphate Buffered Saline (pH 7.2). The developing solution TMB-H_2O_2 (Aristrogen, BCL032) was loaded to all the wells at 50 µl/well volume. The plate was incubated for 30 min in dark. The reaction was stopped by adding 25 µl/well of 2N H_2SO_4 to all the wells. The OD was taken at 450 nm. For all the next assays the same sandwich ELISA protocol is followed with the changes mentioned in the respective sections.

Establishing High Fat Diet C57BL/6 mice model

C57BL/6 male mice with Age: 4-6 weeks old, Body weight: 15-21 g, Basal Glucose values: 125-150 mg/dL were Grouped into 6 animals per group. Ethical committee clearance no Invivo/034 issued from Invivo Biosciences.

Group 1: High Fat Diet- HFD with the following formulation [Vanaspati and Coconut oil (3:1 v/v) fed 3 ml/kg body weight per day along with 25% fructose in drinking water] (22).

Group 2: High Fat Diet+Plant extract- HFD+PE with the following formulation [Vanaspati and Coconut oil (3:1 v/v) fed 3 ml/kg body weight per day along with 25% fructose in drinking water] along with 300 mg/kg bodyweight of the methanolic soxhlet extract of leaf.

Group 3: Normal diet- ND with normal rodent chow feed.

Blood was collected between 10 AM to 12 PM, On day 0, week 4, 8, 12 and 16 around 100 µl of blood was collected from which ~ 50 µl of serum was obtained. The weight of the animals and the blood glucose levels were recorded on the day of every blood collection.

Sample analysis by ELISA

Serum samples from normal diet, high fat diet and high fat diet with plant extract mice diluted at 1:10 were taken for ELISA to quantify the amount of TNF alpha protein present in them.

Statistical analysis

The experiment was carried out in duplicates and results were expressed as mean ± SD for phytochemical analysis and mean ± SEM for animal studies. Statistical significance was analysed using Student's t-test (*$p < 0.05$). Confidence of interval was calculated by GraphPad Prism 5 Software.

RESULTS

Extraction

The yield was higher in leaf (13.2 %) than bark (11.5 %) when extracted through Soxhlet in methanol.

Qualitative and quantitative phytochemical composition

Preliminary qualitative screening of the phytochemicals in *H. antidysenterica* revealed the presence of flavonoids, tannins, alkaloids, glycosides, saponins, carbohydrates, proteins, fats, phenolic compounds, terpenoids and steroids as given in supplementary data, Fig. S1 (a-h) and table S1. Quantitative estimation was performed for selected phytochemicals as given in table 2 and in Fig. 2 (a-e).

Table 1: Content of phytochemical compounds in methanolic extract of leaf and bark

Phytocompounds	Leaf	Bark
Flavonoids (mgQE/g extract)	47.68 ± 0.272	28.22 ± 0.790
Tannins (mgTA/g extract)	27.66 ± 0.209	4.07 ± 0.351
Total phenol content (mg GAE/g extract)	2.1 ± 0.007	1.21 ± 0.003
Alkaloids	0.52	0.12
Proteins and amino acids (mgBSA/g extract)	1.187 ± 0.115	0.533 ± 0.005
Total fat	10	220

Values are mean of triplicate determination (n =2) ± standard deviation; GAE-Gallic acid equivalents; QE-Quercetin equivalents; TA-Tannic acid equivalents; BSA-Bovine serum albumin equivalents.

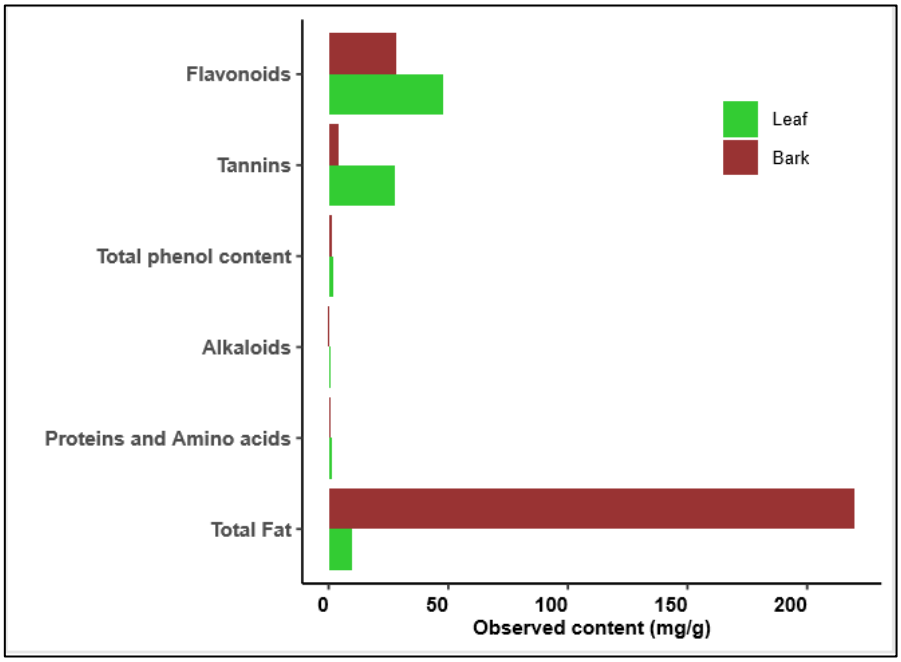

Fig. 2: Estimation of phytochemicals in methanolic extracts of bark and leaf of the plant

Antioxidant property

In this study, DPPH method was used to analyse the antioxidant activity of leaf and bark of *H. antidysenterica*. It was found that both leaf and bark extracts of *H. antidysenterica* showed stronger DPPH scavenging activities (Fig. 3). When the radical scavenging activities of the leaf and bark were compared, bark was found to have higher DPPH (IC_{50} value: 9.27 µg/mL; 95% CI: 7.41 - 11.60) followed by Leaf (IC_{50} value: 6.55 µg/mL; 5.23 - 8.21). Ascorbic acid (IC_{50} value: 1.38 µg/mL; 1.26 - 1.52) was used as standard in this study.

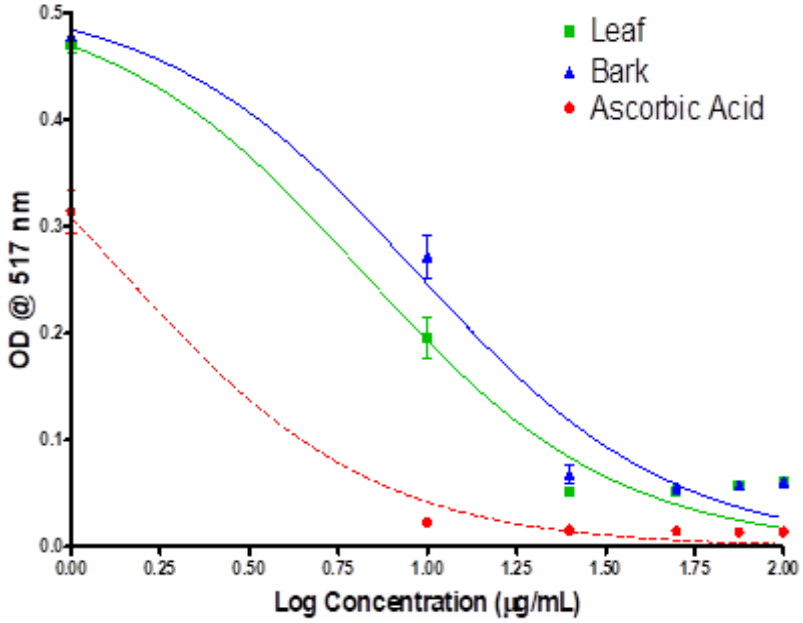

Fig. 3: 4PL graph plot for the calculation of IC_{50}.

Polyclonal antibody generation

A yield of 16.94 mg of antibody was obtained from 1 ml antisera. The purified antibody was run on SDS-PAGE for purity check where two bands corresponding to IgG heavy and light chain was observed at ~ 50 and 25 kDa respectively. Results are given in supplementary Fig. S3 (a-c). This purified antibody was conjugated to HRP and take for ELISA as detection.

ELISA assay establishment and validation

The standardisation of assay parameters is detailed in supplementary section 2 (table S2-S5 and Fig S4-S6). The three standard curves each performed with triplicate readings show that the assay is reproducible with in the validation range of 3 to 0.0012 µg/ml. The established ELISA assay was validated and was ready to be used for sample analysis. The raw data is given in supplementary table S6 and the validation plots are given in Fig 4.

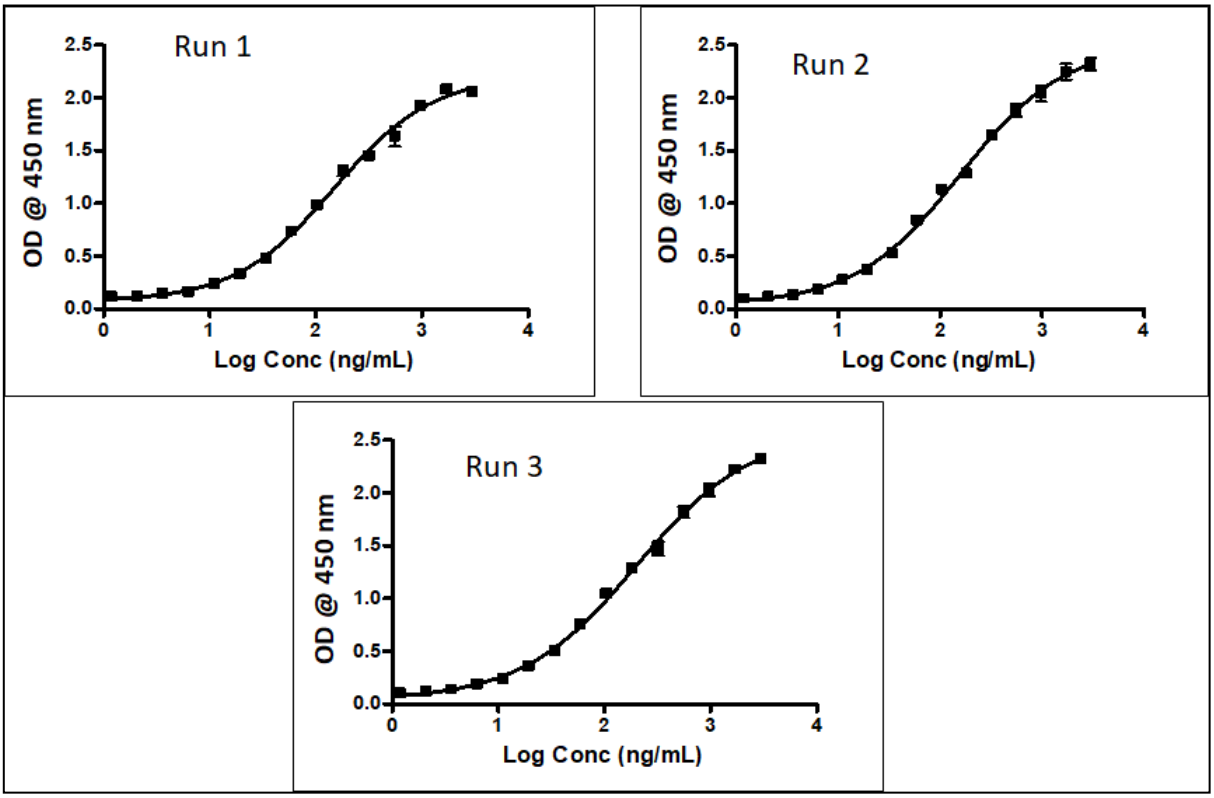

Fig. 4: Graph plot for the validation runs.

TNF alpha levels in High Fat Diet C57BL/6 mice model

There was no difference in the body weight observed among the 3 groups. No difference in the glucose values observed between ND and HFD groups. The glucose values in the HFD+PE group started showing a decline after 8 weeks of treatment, which followed up to 16th week. The maximum glucose dip in the HFD+PE group was observed at 12th week up to 111 ± 2.9 mg/dl from 130.83 ± 6.43 mg/dl in HFD group. TNF alpha levels were higher in HFD group on week 12 with 29.76 ± 5.1 mg/dl compared to ND with 24.86 ± 1.63 mg/dl. TNF alpha levels were not different till 8 weeks of treatment. Only on 12th week there was decrease in the TNF alpha levels observed in HFD+PE group up to 22.08 ± 2.08 µg/ml from 29.76 ± 5.1 µg/ml in the HFD. The graph plots of the parameters between the groups for week 12 is given in Fig. 5. The raw data of body weight, glucose values and TNF alpha levels is given in supplementary table S7.

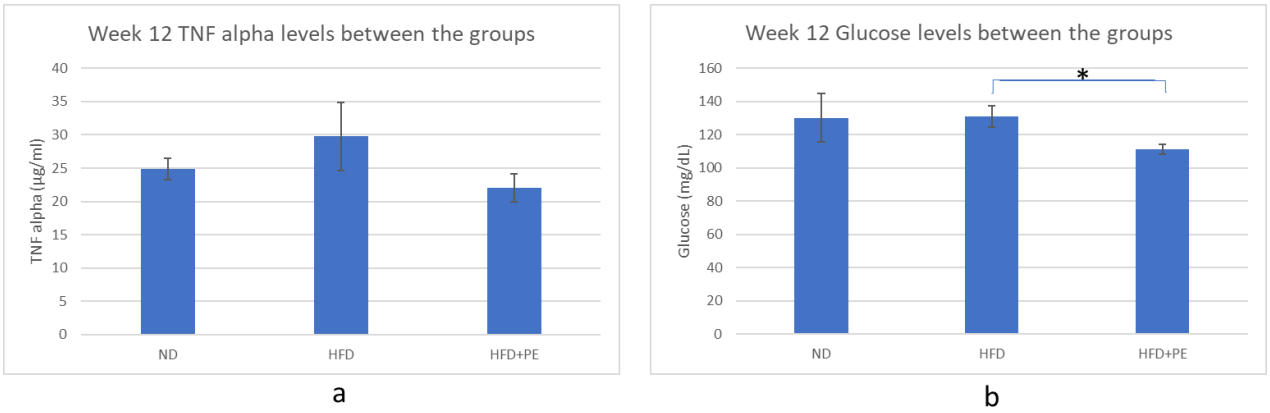

Fig. 5: (a) TNF alpha levels between groups on week 12 (b) Glucose levels between the group on week 12 * p<0.05.

DISCUSSION

Antioxidant and antidiabetic activity of plants are mediated by selected primary and secondary metabolites. Primary metabolites include sugars, fatty acids, amino acids and nucleic acids. Phenols, flavonoids and tannins are the secondary metabolites. It is well-known that plant parts extracted in methanol has higher antioxidant activity due to the presence of phenols, flavonoids, and polyphenols (23).

The leaf extract of *H. antidysenterica* have higher amounts of flavonoids, alkaloids and phenols (Fig. 2), hence conferring its antioxidant property. However, the presence of fat in bark might play a role to improve the bio-availability of active phytocompounds, thus enhancing its antioxidant activity (24).

The developed Sandwich ELSIA was validated according to the guidelines (20) with the detection limit of 0.011 µg/ml.

The High Fat Diet did not show any difference in the body weight but there was an increase in TNF alpha levels observed with marginal increase in the blood glucose levels when compared to normal diet. Upon administration of leaf extracts of *H. antidysenterica* there was a decrease in TNF alpha levels and glucose values as indicated in Fig. 5. This HFD model is close to the life style induced hyperglycaemia (23).

TNF alpha plays an important role in induction of diabetes and hyperglycaemia so inhibiting that gives a hope in the treatment of the disease or reducing the severity. Inhibiting TNF alpha will reduced the chances of developing insulin resistance shown by a case study (25). Oxidative stress and increased calory intake are the two major problems of diabetes. Excess of TNF alpha leads to serine phosphorylation of IRS2, inhibiting the tyrosine phosphorylation of insulin receptor, which has to happen upon insulin binding to the receptor. When tyrosine phosphorylation is absent, the downstream AKT pathway gets inhibited by not converting PIP2 to PIP3, hence GLUT4 is not getting translocated to the plasma membrane so glucose in not transported inside the cell. By reducing TNF alpha levels we can set this pathway right and increase the glucose uptake (26). This is shown in our *in vivo* model where TNF alpha and blood glucose are reduced upon treatment with leaf extract of *H. antidysenterica* as in Fig. 5.

This is a very preliminary study to know the molecular mechanism involved in the anti-diabetic effect on *H. antidysenterica*. Crude leaf extracts of *H. antidysenterica* showed a decrease in TNF alpha and blood glucose levels in the mice. Indicating in the reversal of insulin resistance which was induced by high fat diet. Phenols, Flavonoids and Tannins are the abundant phytochemicals found when leaf extracted in methanol as shown in Fig. 2 these phytochemicals have anti-diabetic activity. Further work in the direction of finding out the active compound from the leaf extracts by activity guided fractionation is needed to pin point the actual molecule(s) responsible.

CONCLUSION

By DPPH assay, it can be concluded that *H. antidysenterica* shows high potency as a scavenger of hydroxyl radicals. Phenolic compounds conferring antioxidant activity to plant parts are likely to be more bio-accessible in bark in presence of elevated fat content, thus resulting in its antioxidant potency. Leaf methanolic extract of *H. antidysenterica* has a promising effect in reducing the TNF alpha and blood glucose levels in HFD animals. There is a lot of scope for the future work on molecules involved in anti-diabetic activity of *H. antidysenterica*.

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CONFLICT OF INTEREST

The authors declare they have no competing interests.

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