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Research article

ANTIGLYCATION, ANTIOXIDANT AND ANTIDIABETIC ACTIVITY OF TRADITIONAL MEDICINAL PLANT: *RUBIA CORDIFOLIA* LINN. FOR MANAGEMENT OF HYPERGLYCEMIA

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ABSTRACT: *Rubia cordifolia* Linn. a flowering plant species from family Rubiaceae is an important medicinal plant. It is known to have anti-diabetic, antiseptic and antistress properties. In the present paper, antiglycation, antioxidant and antidiabetic activities of roots of *Rubia cordifolia* Linn. were investigated. Anti-AGE (Advance glycation end products) effect of *Rubia cordifolia* Linn. on glycation of guanosine with glucose and fructose was analyzed. The inhibitory effect of plant extract on glycation and fructation of guanosine in presence of reactive oxygen species (ROS) generated by hydrogen peroxide, was also analyzed. *In vitro* antioxidant (DPPH, Superoxide anion scavenging activity and Xanthine oxidase) and antidiabetic assays (Alpha amylase and alpha glucosidase) were performed to determine these activities of plant extract. The UV absorbance of the glycation reactions was found to be maximum at 24 hrs. and with consecutive decreased at 48 and 72 hours. Plant extract of *Rubia cordifolia* Linn. inhibits glycated and fructated guanosine and ROS-modification of glycated and fructated guanosine. From the results, it is concluded that root extract of *Rubia cordifolia* has significant antiglycation, antioxidant and antidiabetic activities. The study suggests that *Rubia cordifolia* Linn. is a natural antioxidant, which might be helpful in management of diseases like diabetes.

Keywords: Antioxidant activity, α-Amylase, α- Glucosidase, Antiglycation

INTRODUCTION

Rubia cordifolia Linn. (Rubiaceae), also known as 'Manjistha', is an important medicinal plant and is used for treatment of various ailments like diabetes, cancer etc.. Anti-hyperglycemic and antioxidant properties of the roots of *Rubia cordifolia* Linn. have been reported earlier [1] while aerial parts of the plant also show hypoglycemic effect [2]. The importance of decreasing Advanced glycation end products (AGEs) accumulation as a potential therapeutic target in diabetic nephropathy has been clearly demonstrated by experimental studies using a range of inhibitors of advanced glycation [3, 4]. AGEs are formed as a major factor in diabetes. They are formed *in vivo* through a non-enzymatic glycation of glucose and other reducing sugars with proteins via so-called Malliard reaction. Hyperglycemia and free radical formation are characteristics of diabetes which accelerate the formation of advanced glycation end products (AGEs) [5]. Glycation affects all major classes of biomolecules with damage estimated at 0.1–1% of lysine and arginine residues on proteins, 1 in 107 nucleotides on DNA and 0.1% of basic phospholipids [6]. It has been shown that DNA nucleotides can be efficient glycation targets [7]. DNA glycation has received remarkable attention and number of DNA AGEs have been identified [7,8]. N2 carboxyethyl guanosine (CEG A, B) and carboxy ethyl guanine (CE guanine) are analogus derivatives formed during glycation of guanosine or guanine respectively [9]. So, it was of interest to investigate the inhibitory action of plant extract of *Rubia cordifolia* Linn. on the glycated and fructated guanosine. In view of the wide occurrence of diabetes, a great demand exists for the antiglycation agents (AGEs inhibitors), especially from the natural sources as adverse side effects are associated with potential synthetic drugs [10]. According to Reddy and Beyaz (2006), antioxidants act as AGEs inhibitors. Antioxidants inhibit or slow down scavenging free radicals providing protection against many chronic diseases [11]. Naturally occurring antioxidants from fruits, leaves etc. are of great interest [12, 13].

Therefore, aim of the present study was to determine the antiglycation, antioxidant and anti-diabetic activities of *Rubia cordifolia*. For antioxidant activity three *in vitro* assay systems (DPPH, Xanthine oxidase, Superoxide anion scavenging) and for anti-diabetic activity, two assays (alpha-amylase and alpha-glucosidase) were employed in order to understand the usefulness of plant as a medicine.

MATERIALS AND METHODS

Chemicals

NBT (nitroblue tetrazolium), Xanthine oxidase, Allopurinol, EDTA, Ascorbic acid, hypoxanthine, α-amylase, α-glucosidase, p-nitrophenyl-α-d-glucopyranoside, D-Glucose, D-fructose and hydrogen peroxide were purchased from SRL (India). DPPH (2, 2′- diphenyl-1-picrylhydrazyl), quercetin, guanosine were purchased from Sigma Chemicals Co. All other chemicals and reagents were of analytical grade.

Plant Material

In-vitro grown roots of *Rubia cordifolia* Linn. were used for the study.

Preparation of Plant Extract:

Roots of *Rubia cordiflia* (250 g) were extracted using methanol (250 ml) in Soxhlet apparatus for 8 hours. Then, the extract was evaporated to dryness and the final dry crude extract was stored in dark at -20°C until used for the experiments.

Modification of Guanosine with Glucose and Fructose

Guanosine (100 µg) was modified with glucose and fructose (600 mg) and was incubated under sterile conditions for 24, 48 and 72 hours at 37°C. Solution of DNA without guanosine served as a control.

Modification of Glycated and Fructated Guanosine with Hydrogen Peroxide

The aqueous solution of glycated and fructated guanosine was treated with 100 fold excess of hydrogen peroxide (mM) and was kept for incubation at 37°C for different time periods. After incubation for 24, 48 and 72 hours, the tubes were irradiated under 254nm UV light for 30 minutes. The samples were then scanned for UV absorption spectra using Shimazdu UV-240 spectrophotometer.

ANTIOXIDANT ASSAYS

DPPH- Free Radical Scavenging Assay

Free radical scavenging activity of the extract was measured using stable DPPH (2, 2′- diphenyl-1-picrylhydrazyl). Dilutions of plant extract (2-80 µg/µl) were prepared before analysis. 40 µl of the diluted sample or control was placed in appropriate wells of the microtiter plate and 80 µl of Tris-HCl buffer (50mM, P^H 7.4) was added. 120 µl of DPPH solution (0.2 mM in absolute ethanol) was then added and the solution was mixed well by pipetting. The mixture was then incubated at ambient temperature (25°C) in the dark for 30 minutes. The free radicals scavenging potential of extracts was evaluated spectrophotometrically at 517 nm. Ascorbic acid was used as a standard compound (2-80 µg/ µl concentration range). DPPH solution in a reaction was replaced with ethanol for blank. Blank was included for each sample dilution. The percentage inhibition was calculated using the following formula:

% Inhibition= [(OD Control – OD Sample)/ (OD Control)] x 100

Superoxide Anion Scavenging Activity

Super oxide scavenging activity was determined by NBT reduction method of [14]. The test implements two principal reactions:

$$2NBTH \longrightarrow NBT + NBTH_2 \text{ (Formazon)}\ldots\ldots \text{ (a)}$$

$$NBTH + O_2 \longleftrightarrow NBT + O_2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{ (b)}$$

Formazon formed in reaction (a) reacts with antioxidants that donates an electron to NBT. In donating an electron, purple colour of formazon disappears which can be followed by spectrophotometer at 560nmVarious concentrations of plant extracts were pre-incubated at room temperature with the reaction mixture of 20 µl Na_2EDTA (15mM) in Na_2HPO_4 - KH_2PO_4 buffer (66.67mM PH-7.5), 50 µl NBT (0.6mM), 30 µl of 3mM hypoxanthine in Na_2HPO_4 - KH_2PO_4 buffer. 50 µl of 1 U in 10ml buffer xanthine oxidase solution was then added to the mixture. The tubes were exposed to light for 15 minutes and optical density was measured at 570nm after the illumination. Percent inhibition was measured using the following formula:

% Scavenging = [(OD Control – OD Sample)/ (OD Control)] x 100

Xanthine Oxidase Assay

Xanthine oxidase is a terminal enzyme of Purine catabolism. It catalyzes the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid.

The following chemical reactions are catalyzed by xanthine oxidase:

- hypoxanthine + H_2O + O_2 ⟷ xanthine + H_2O_2
- xanthine + H_2O + O_2 ⟷ uric acid + H_2O_2
- Xanthine oxidase can also act on certain other purées, pterins, and aldehydes. For example, it efficiently converts 1-methylxanthine (a metabolite of caffeine) to 1-methyluric acid, but has little activity on 3-methylxanthine.
- Under some circumstances it can produce superoxide ion $RH + H_2O + 2\ O_2 \leftrightarrow ROH + 2\ O_2^- + 2\ H^+$.

Assay was based on the procedure reported by [15]. The inhibitory effect of Xanthine oxidase was measured spectrophotometrically at 295nm. Allopurinol was used as a positive control. The reaction mixture contained a varying concentration of plant extract mixed with Na_2HPO_4-KH_2PO_4 buffer (66.67mM, pH 7.5, 1.3ml) and Xanthine solution (0.15mM in ddH_2O, 1.5ml). This mixture was then incubated at 30°C for 10 minutes. Xanthine oxidase solution (0.28U/ml in 66.67 mM phosphate buffer) was added to the mixture to initiate the reaction. The absorbance at 295nm was measured each minute for 10 minutes using a microtiter plate reader. The % inhibition of this enzyme with plant extracts was calculated using the equation below:

% Inhibition= [(OD Control – OD Sample)/ (OD Control)] x 100

Determination of Flavonoid Content

Total flavonoid content was determined by an assay described by [16]. The reaction mixture contained various concentration of plant extract (10-100 µg/ µl), 75 µl of 5% $NaNO_2$ and 150 µl of 10% $AlCl_3$. Final volume of the reaction was adjusted to 2.5 ml with distilled water. After mixing and incubation for 5 minutes, 500 µl of 1M NaOH was added to the reaction. The absorbance was measured at 510nm.Quercetin (0.5mg/ml) was used as a standard. Flavonoid content was expressed according to the following formula:

% Flavonoids= [{(OD extract * 0.05)/ OD Quercetin}/ Extract concentration] *100

Antidiabetic Assays

Insulin secretion in diabetes can be enhanced by antidiabetic agent. The present study represents roots of *Rubia cordifolia* as a potential hypoglycemic agent.

α-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined by an assay modified from the Worthington Enzyme Manual [17]. A total of 500µl of sample extract and 500 µl of 0.02M sodium phosphate buffer (pH 6.9 with 0.006M NaCl) containing α-amylase solution (0.5mg/ml) were incubated at 25°C for 10 minutes. After pre-incubation, 500 µl of a 1% starch solution in 0.02M sodium phosphate buffer (pH 6.9 with 0.006M NaCl) was added to each tube at timed intervals. The reaction was stopped with 1ml of dinitrosalicylic acid (DNS) color reagent. The test tubes were incubated in a boiling water bath for 5 minutes and cooled to room temperature. The reaction mixture was then diluted after adding 5-15ml distilled water, and the absorbance was measured at 540nm using UV-visible spectrophotometer. The readings were compared with the controls, containing buffer instead of sample extract. The percentage α-amylase inhibitory activity was calculated using the following formula:

% Inhibition= [(OD Control – OD Sample)/ (OD Control)] x 100

α- Glucosidase Inhibition Assay

The α- Glucosidase inhibitory activity was determined by an assay modified from [18]. α- Glucosidase was assayed by using 50 µl of sample extracts and 100 µl of 0.1M phosphate buffer (pH6.9) containing α- glucosidase (1U/ml) and was incubated in 96 well plates at 25°C for 10 minutes. After pre-incubation, 50 µl of 5mM p-nitrophenyl-α-d-glucopyranoside solution in 0.1M phosphate buffer (pH6.9) was added to each well at timed intervals. The reaction mixture was incubated at 25°C for 5 min. Before and after incubation, absorbance readings were recorded at 405nm by a microplate reader and compared to a control that had 50 µl of buffer solution in place of extract. The α- glucosidase inhibitory activity was expressed as percentage inhibition and was calculated using the formula:

% Inhibition= [(OD Control – OD Sample)/ (OD Control)] x 100

Statistical Analysis

All samples were analyzed in triplicate. Data are presented as mean ± standard error.

RESULTS AND DISCUSSION

Glycation of Guanosine and ROS-Modification of Glycated and Fructated Guanosine

Like protein, DNA contains amino groups. The 2-amino groups of guanosine are the most reactive. Reaction of glucose with guanosine yields W-glucopyranosylguanosine [19]. Both the Amadori product and later-stage AGES undergo auto-oxidation and have pro-oxidant effects on other molecules [20].

In maximum absorbance of glycated and fructated guanosine was observed at 24 hours and it goes on decreasing at 48 and 72 hours respectively (Figure 1a, b). This decrease in absorbance may be due to the blockage of free NH_2 group present in guanosine as a result of non-enzymatic glycation. Figure 1c, d displays the results of ROS-modified glycated and fructated guanosine. The absorbance is continuously increased from 24 to 72 hours which may to be due to breakage of bonds and bases as a result of oxidative damage leading to this hyperchromic effect.

Antiglycation Inhibition Activity of Plant Extract with Glycated and Fructated Guanosine

Plant extract of *Rubia cordifolia* Linn. showed inhibition activity of 11%, 12%, 8.30% at 24, 48 and 72 hours respectively on fructated guanosine (Figure 2 a). Inhibition activity is decreased by % at 72 hours which indicates maximum inhibition at 48 hours. Plant extract of *Rubia cordifolia* Linn. did not inhibit glycated guanosine at tested concentrations of extract.

Antiglycation Inhibition Activity of Plant Extract with ROS-Modified Glycated And Fructated Guanosine

Plant extract of *Rubia cordifolia* Linn. inhibits glycation of ROS-modified glycated guanosine when reacted with hydrogen peroxide. Increase in the inhibition of plant extract and decrease in absorbance of ROS-modified glycated guanosine is observed as a result of oxidative damage (Figure 2b). Inhibition activity of plant extract with glycated guanosine is further increased when 100µg of plant extract was added to the reaction mixtures at 48 and 72 hours. Inhibition of 12% and 18% is observed as a result of oxidative damage at 48 and 72 hours respectively in case of ROS-modified glycated guanosine. No inhibition of plant extract was observed at 24 hours for ROS-modification of glycated guanosine.

Inhibitory activity of plant extract towards ROS mediated oxidative damage to fructated guanosine is shown in Figure 2c. Decrease in absorbance at different time periods and significant increase in the inhibition activity is observed as a result of oxidative damage. Effect of dosing of 100µg/ml is observed at 48 and 72 hours. Inhibition of 3%, 18% and 29% is observed at 24, 48 and 72 hours respectively as a result of oxidative damage. Plant extract of *Rubia cordifolia* Linn. did not inhibit glycated guanosine as Glucose is least reactive among all sugars [21]. This slow reactivity coupled with time bound activity of plant extract may contribute towards the lack of inhibition of glycation of guanosine. *In vitro* studies suggest that fructose, compared with glucose, is a much more potent initiator of the Maillard reaction [22, 23] and Maillard reaction may be involved in the aging process [24].

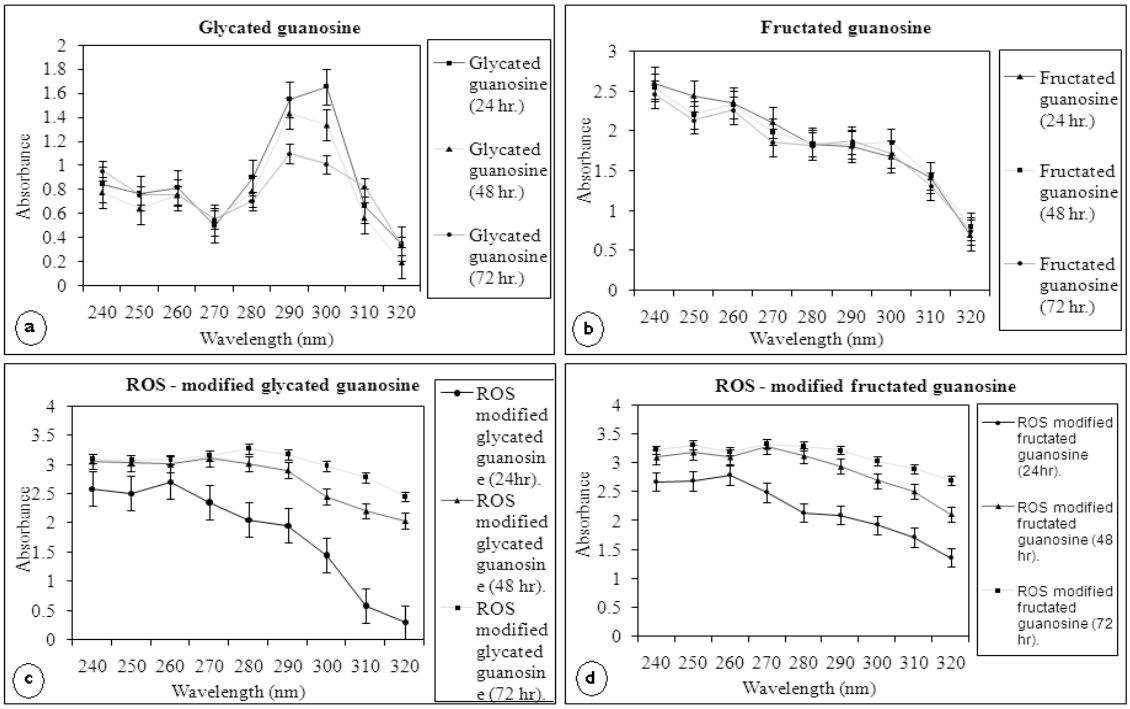

Figure 1: UV Profile of glycated guanosine (a), fructated guanosine (b), ROS-modified glycated guanosine (c), ROS-modified fructated guanosine (d) incubated for 24, 48 and 72 hours. ROS modification was done using hydrogen peroxide.

Plant extract of *Rubia cordifolia* Linn. inhibits fructated guanosine to a maximum of 12% at 48 hours which indicates that the glycation reaction of guanosine with fructose may be comparatively fast as compared to glycation reaction with glucose and thus reflecting the inhibitory effects of the plant extract on this reaction, rather than that with glucose.

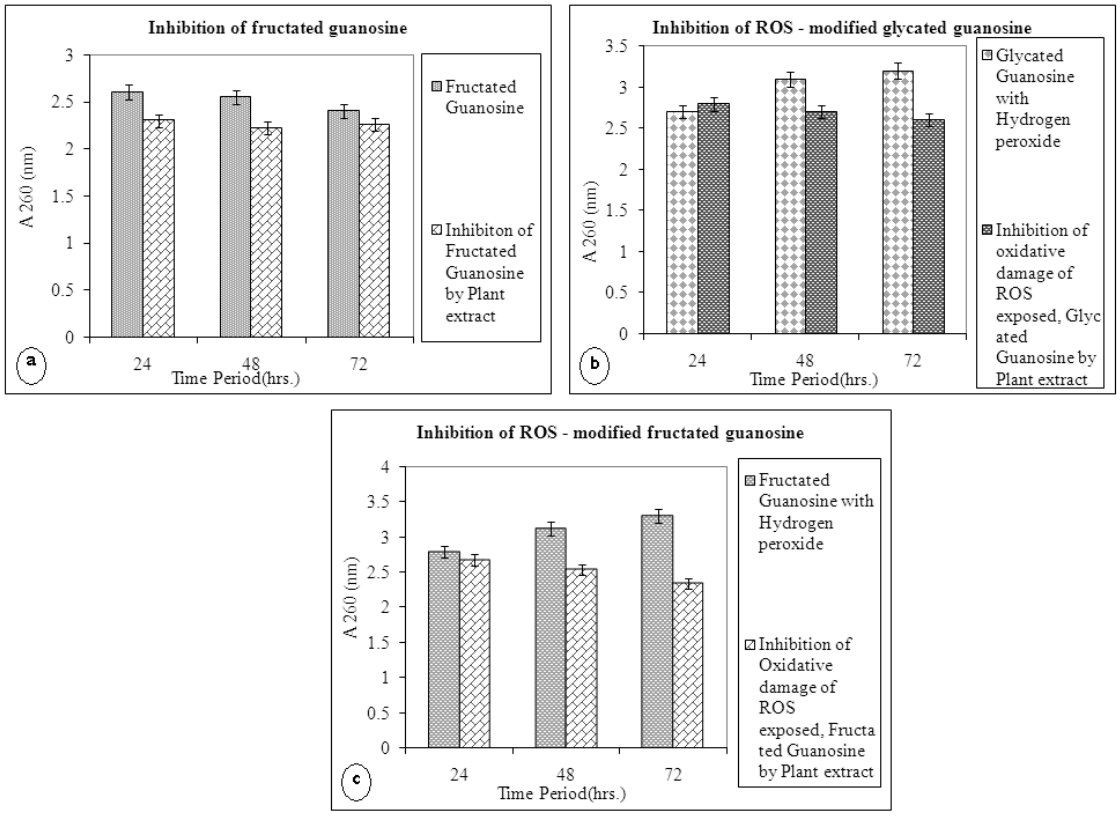

Figure 2: Bar diagram showing inhibition of fructation of guanosine (a), ROS-modification of glycated guanosine (b) and ROS-modification of fructated guanosine (c) by extract of *Rubia cordifolia.*

Plant extract of *Rubia cordifolia* Linn. delays the action of non-enzymatic glycation reactions and further may delay the formation of advanced glycation end products in the form of fructated guanosine.

The observation from our study suggests that plant extract of *Rubia cordifolia* Linn. has the potential to act as an antiglycating agent. Therefore our finding of effective inhibition of free radical with hydrogen peroxide by the plant extract of *Rubia cordifolia* Linn. in ROS-glycated and fructated guanosine is an important finding especially from therapeutic point of view. Maximum inhibition of 18% and 29% is seen in ROS modification of glycated and fructated guanosine at 72 hours. ROS exposed fructated guanosine is seen to be most potent inhibitory target of the plant extract as fructose is the potent initiator in the Malliard reaction as compared to glucose.

ANTIOXIDANT ASSAYS

DPPH- Free Radical Scavenging Activity

DPPH method is extensively used for the estimation of free radical scavenging activity of variety of samples [25]. DPPH is characterized as nitrogen centered free radical. Because of strong absorption band at 517nm, DPPH radical has a deep violet color in solution which changes to yellow due to the delocalization of the spare electron over the molecule. IC_{50} value of root extract of *Rubia cordifolia* Linn. and control ascorbic acid was found to be 2.39µg and 1.59µg respectively. Maximum percent inhibition by the extract of *Rubia cordifolia* Linn. was observed to be 71% at a concentration of 9µg ±1.07 as compared to ascorbic acid which was observed to be 79% at a concentration of 9.17µg ±1.04.

Total Flavonid Content

Phenols and flavonoids in plant have been shown to possess antioxidant activities. % Flavonoid content in terms of quercetin equivalent of *Rubia cordifolia* Linn. was found to be 38.33mg/100mg.

Superoxide Anion Scavenging Activity

Superoxide is produced by the enzyme NADPH oxidase, and contributes to the pathogenesis of many diseases. Superoxide anions are formed from dissolved oxygen by the coupling reaction which reduces NBT in the reaction. Consumption of superoxide in the reaction is monitored by the decrease in absorbance at 560nm with extract of *Rubia cordifolia*. Maximum percent inhibition of *Rubia cordifolia* Linn. root extract on superoxide free radical was found to be 56% at a concentration of 600µg. Standard quercetine showed a maximum inhibition of 72% at a concentration of 756µg.

The IC_{50} value for superoxide scavenging activity of root extract of *Rubia cordifolia* Linn. was found to be 551.96 ±1.24 whereas, the IC_{50} value of the standard Quercetin was 128.21± 2.31.

Screening of Root Extracts of Rubia cordifolia Linn. For Xanthine Oxidase (XO) Inhibitory Activity

Xanthine oxidase plays a major role in the purine nucleotide metabolism in humans. Its major function is to catalyze the oxidation of hypoxanthine to xanthine and of xanthine to uric acid [26] .Overproduction of uric acid leads to hyperuricemia which is linked to gout [27]. Herbal remedies obtained from traditional herbs and medicinal plants are commonly used these days for treating various diseases. XO generates superoxide, in presence of NADH oxidase which is a Reactive oxygen species (ROS). Xanthine oxidase is seen to be produced during ischemia-reperfusion injury, leading to cell degeneration and death. Figure 3 shows the Xanthine oxidase inhibitory activity of root extracts of *Rubia cordifolia*. Maximum of 49.17% Xanthine oxidase inhibitory effect at 500 µg plant extract was observed. Standard Allopurinol (500µg) showed a maximum inhibition of 73.46%.

Inhibition Activity of α- Amylase and α-Glucosidase

Alpha amylase and glucosidase inhibitors, the digestive enzymes are considered to be effective for the treatment of diabetes, obesity and hyperlipaemia [28]. It is important to identify the properties and biological effects of naturally occurring inhibitors from plants and other sources which are used for the treatment of diabetes [29].The medicinal plants or natural products involve retarding the absorption of glucose by inhibiting the carbohydrate hydrolyzing enzymes. Several inhibitors including acarbose, voglibose and miglitol are used but their prices are high [30]. Figure 4 (a) and 4 (b) displays the alpha amylase and alpha glucosidase activity of root extract of *Rubia cordifolia*. In amylase assay the positive control acarbose showed an IC_{50} 47.98 ± 1.2 and plant extract of *Rubia cordifolia* Linn. showed an IC_{50} 95.34 ± 1.6. Maximum percent inhibition of *Rubia cordifolia* Linn. plant extract and Positive control acarbose was observed to be 50% and 69% at a concentration of 98.33 ± 1.54 µg and 96.67 ± 2.89 µg respectively.

In Alpha glucosidase assay IC_{50} of control acarbose and plant extract was found to be was found to be 47.98 ± 1.04 and 95.34 ± 1.6 respectively. Maximum percent inhibition of acarbose and plant extract was observed to be 69% and 50% at a concentration of 96.67 ± 2.89 µg and 98.33 ± 1.54 µg respectively.

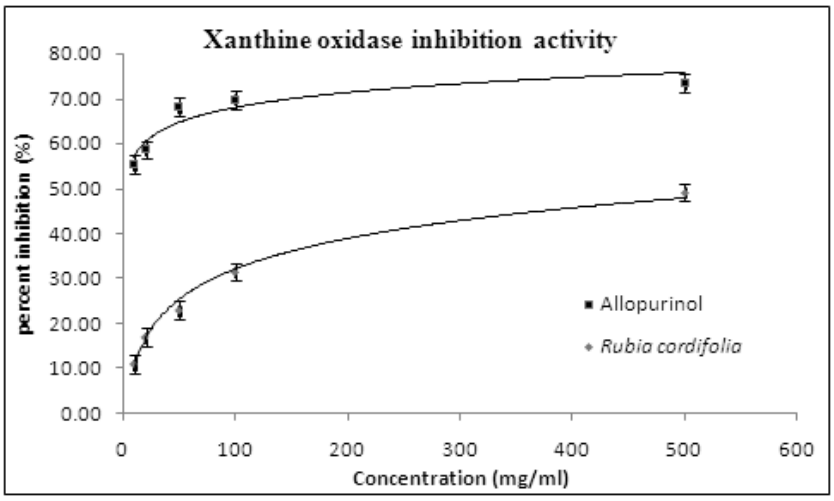

Figure 3: Xanthine oxidase inhibitory activity (%) of methanolic extract of *Rubia cordifolia* Linn. on DPPH radicals. Positive control was Allopurinol. Each value is expressed as mean ± standard deviation of triplicate experiments.

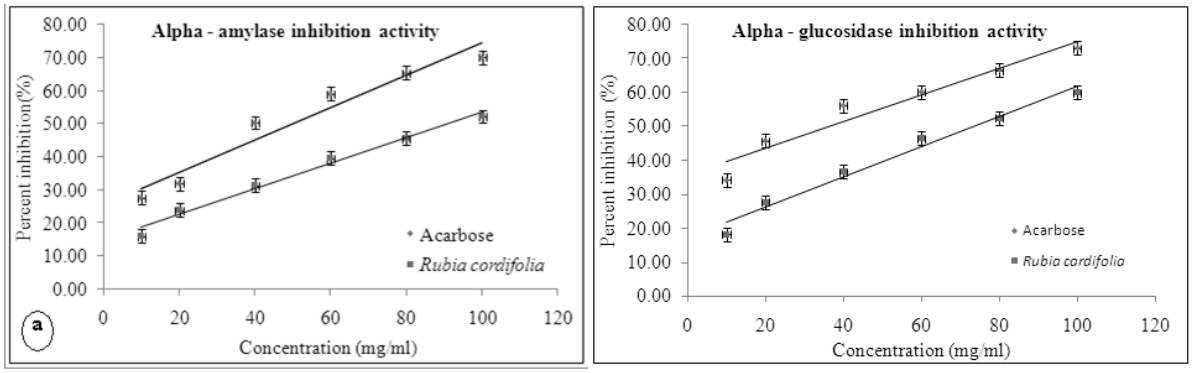

Figure 4: Alpha amylase (a) and Alpha glucosidase (b) inhibition by plant extract of *Rubia cordifolia.* Each value represents mean ± standard deviation of triplicate experiments.

CONCLUSION

The present study demonstrated the anti-diabetic potential of *Rubia cordifolia* along with a significant antiglycation and antioxidant activity indicating its potential to be used as an antidiabetic drug for management of hyperglycemia. Isolation, purification and characterization of the compound(s) responsible for inhibiting activity is under progress.

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CONFLICT OF INTEREST

Author declare no conflict of interest.

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