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Anti-diabetic Activity of Partially Purified Santalin A from the Heartwood of *Pterocarpus santalinus* L.f. in Alloxan-induced Diabetic Wistar Rat

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Abstract

The ever-increasing use of plant-based pharmaceuticals as alternatives to conventional drugs for disease management demands identification, isolation, and characterization of novel compounds. Despite the potential of plant extracts to mitigate the morbidity of diseases, several active principles are preferred to avoid the interference of other compounds. The promising health benefits of the extracts and isolated compounds of *Pterocarpus santalinus* in the treatment of diabetes, cardiovascular disease, cancer, and infections have been described. However, such studies on the active principle, namely, santalins, are not reported. In this study, we standardized the isolation of a mixture of santalins A and B from the heartwood of *P. santalinus* by column chromatography followed by preparative TLC and HPLC. The partially purified santalins were characterized by LC-MS, HR-MS, and ^{1}H NMR analyses. The isolated combination of santalins displayed higher total antioxidant and DPPH free radical scavenging activity *in vitro* than the crude heartwood extracts. Administration of the mixture of santalins A and B did not exhibit any antihyperglycemic activity in the liver, kidney, and pancreas of alloxan-induced diabetic rats. However, pretreatment of rats with a mixture of santalins at a dose of 1.0 mg/kg body weight prevented alloxan-induced diabetes as indicated by the normal blood glucose levels. Hyperglycemia-associated lipid peroxidation was abrogated in santalin-pretreated rats that did not develop alloxan-induced diabetes. Furthermore, the alterations in catalase, glutathione peroxidase, and glutathione-S-transferase activities in the pancreas of santalin-pretreated rats could be responsible for preventing damage to the pancreas and thus non-induction of diabetes following alloxan treatment. Therefore, for the first time, we report the simplified procedure for isolating a mixture of santalins, including their ability to prevent the induction of diabetes in Wistar rats. The outcome of our study has significant clinical importance to the fact that supplementation of santalins may potentially avoid or delay the onset of diabetes in high-risk individuals.

Keywords: Antidiabetic, Antioxidant, Oxidative Stress, Red Sanders, Santalins

1. Introduction

Medicinal plants used in Indian ayurvedic traditional systems to treat various diseases are of immense value. They constitute a vital element of the Indian health care system since ancient times[1]. Recent trends indicate that studies to identify the health benefits of medicinal plants are on the rise because of the constant demand to identify newer molecules for health benefits[2]. Concomitantly, the international trade in materials derived from medicinal plants is also increasing[3]. The phytocompounds have become more popular than conventional pharmaceuticals due to their fewer side effects. The compounds isolated from the

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medicinal plants possess functional properties such as antibacterial, antiviral, antihypertensive, anticancer, and antidiabetic activities[4-8]. Among many plants that are anticipated to have health benefits, some are also known to have cosmetic value[9-12]. Research on identifying newer phytocompounds from such plants and deciphering their functional role in health benefits is an active area of investigation in the current times.

Pterocarpus santalinus L.f., commonly known as red sandalwood or red sanders, is an economically important tree species belonging to Fabaceae. In India, the heartwood of red sanders is used for making dolls, food colorants, and cosmetic preparations. *P. santalinus* is a precious indigenous tree that is widely used to treat various ailments due to its extensive medicinal properties (Bulle *et al*., 2016)[13]. Different plant parts of this species have been used in powder, paste, and decotion to treat fever, headaches, diabetes, and treat inflammatory diseases such as chronic bronchitis[14]. Narayan *et al.* (2005)[15] reported its use as an herbal drug for the treatment of NSAID-induced gastric ulcers. Thus, the researchers have an upsurge of interest in exploring the phytochemistry, pharmacology, and ethnomedicinal values of this tree species[14-16]. Several research groups have studied the antidiabetic, antioxidant, antibacterial, anti-inflammatory, hepatoprotective, angiogenic, and analgesic activities of plant extracts with promising results[17-20]. The different effects exhibited by the extracts of this plant are believed to be due to the presence of various bioactive compounds that belong to phenols, alkaloids, saponins, flavonoids, terpenoids, tannins, and complex carbohydrates[14,21]. Compounds that are mainly present in the heartwood extract of this plant are santalins A, B, C, and Y (biflavonoids), pterostilbene (methyl ester of resveratrol), beta-eudesmol, isopterocarpalone, cryptomeridiol, and pterocarpodiolones[13]. Among the many health benefits exhibited by the extracts of *P. santalinus*, their potential use as antidiabetic agents assumes significance in the Indian context because of the high incidence of the disease and morbidity.

Diabetes mellitus is characterized by compromised antioxidant status, increased free radical generation, lipid peroxidation, and susceptibility to infections. The manifestation of diabetes is primarily due to high glucose in the blood, and, therefore, bioactive compounds that can exhibit antihyperglycemic activity are being identified for better treatment strategies[14,21]. Besides the management of glycemia in diabetes, preventing or delaying the onset in high risk individuals is a crucial aspect to avoid diabetic complications. Thus, experimental models that can demonstrate the beneficial actions of bioactive compounds in preventing or delaying the onset of diabetes assume as much significance as the management of diabetic individuals. Although the antidiabetic activity of the extracts of *P. santalinus* was demonstrated, no studies have reported such beneficial effects from the purified santalins. Hence, in this study, we have partially purified santalin A and analyzed its hypoglycemic potential in an alloxan-induced diabetes rat model by adopting two treatment strategies that reflect the management and prevention of diabetes. Although post-diabetes treatment of santalins did not display antihyperglycemic activity in alloxan-induced diabetic rats, pretreatment was efficient in preventing the onset of diabetes. Thus, this study provides evidence that santalins can be used as protective agents for individuals at high risk of developing diabetes mellitus.

2. Materials and Methods

2.1 Samples

The heartwood samples of *Pterocarpus santalinus* L.f. were collected from Seshachalam forest, Chittoor district, Tirupati, Andhra Pradesh, India, and authenticated by Dr. Sateesh Suthari, Young Scientist-SERB. The voucher specimen was deposited in the herbarium (Specimen number 2615) at the Department of Plant Sciences, School of Life Sciences, University of Hyderabad (UH). The collected samples were stored at room temperature until use.

2.2 Preparation of Extract

The heartwood samples were mechanically cut into small pieces and pulverized using sterile mortar and pestle and then blended in a motorized grinder to obtain a fine powder.

2.3 Extraction, Isolation and Partial Purification of Santalins

Santalin A was extracted from the heartwood of red sanders by the sequential methods described earlier[22]. However, we incorporated a few modifications to the isolation procedure to enhance yield. The dried heartwood powder of red sanders of 5 grams was refluxed

with hexane to extract non-polar compounds, followed by petroleum ether and chloroform for 12 hr each to remove lipids. Further extraction was carried out with dichloromethane for 6 hr to remove slightly polar compounds, and the resulting residue was refluxed with methanol for 12 hr, and the filtrate was collected. Color changes in every step were noted, which is an indication of the extraction of different compounds. The filtrate was concentrated by rotary evaporation resulting in a dark red powder.

The powder was placed on a silica gel column (60-120 mesh, 20 g, 100×5 cm) and subjected to gradient elution with increasing polarity of solvents (100% hexane, hexane and dichloromethane (2:0.5, 2:1 and 2:2), 100% dichloromethane (DCM), DCM and methanol (3:0.5, 3:1 and 3:2) and finally 100% methanol. The collected fractions were tested individually by TLC using silica (0.5 mm thickness) as the stationary phase and a solution of DCM and methanol in the ratio 3:1 as the mobile phase. Out of all fractions, 5 fractions from DCM and methanol (3:2) and one fraction from 100% methanol were further subjected to preparative TLC, and the specific compounds in the red color band were recovered by scraping the corresponding sorbent layer from the plate and eluting in 100% methanol. The eluted fraction was then subjected to further purification with preparative HPLC. The identity of the compounds in the purified fraction was confirmed by Liquid Chromatography-Mass Spectrometry (LC-MS) and High-Resolution-Mass Spectrometry (HR-MS), and ^{1}H NMR.

2.4 Total Antioxidant Activity (TAA) of Crude Methanolic Heartwood Extract and Santalins

Phosphomolybdenum method was used for determining the total antioxidant activity of crude methanolic wood extracts of *P. santalinus* and partially purified santalins[23]. The assay is based on Mo (VI) to Mo (V) reduction by the antioxidant compounds and the consequent formation of a green phosphate/Mo (V) complex at acidic pH. The stock solutions of methanolic crude extract and santalins (20 mg/mL) were prepared separately and 10 µL from the stock solution was added to 90 µL of distilled water. To this solution, 1 mL of reagent (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added and incubated in a thermal block at 95°C for 90 min. The solution of each reaction was measured for the absorbance at 695 nm against an appropriate blank after cooling to room temperature. Ascorbic Acid (AA) was used as an internal standard. The total antioxidant capacity was expressed as milligrams of ascorbic acid equivalents/gram dry weight (mg AAE/gDW).

2.5 DPPH Radical Scavenging Activity of Crude Methanolic Heartwood Extract and Santalins

The DPPH radical scavenging activity was assessed based on a previously reported method with minor modifications[24]. DPPH (1, 1-diphenyl-2-picrylhydrazyl) solution (0.004% w/v) was prepared in 95% methanol. Various concentrations (2 to 20 µg/mL) of crude extracts, santalins or ascorbic acid were mixed with 900 µL of 0.004% DPPH and incubated in the dark for 30 min. The color intensity was measured at 517 nm. Appropriate blanks, *i.e.*, DPPH solution alone or AA only, were included. Free radical scavenging activity was calculated according to the following equation:

Free radical scavenging activity (%) = (O.D. of control – O.D. of sample / O.D. of control) × 100.

2.6 Animal Treatments

The experimental procedures for animal studies were approved by the Institutional Animal Ethics Committee (UH/IAEC/PG/2018-I/41) of the University of Hyderabad. Male Wistar rats aged about 75 to 90 days were purchased from Jeeva Bio Labs, Hyderabad, India. Animals had free access to a standard rat pellet feed and water *ad libitum,* housed at ambient temperature and humidity.

Diabetes Type 1 was induced in the rat, as previously described[25]. Each rat was administered intraperitoneally with 250 mg/kg body weight alloxan [prepared freshly in citrate buffer (50 mM, pH 3.0)] for three consecutive days. One week after administration of the first dose, random blood glucose levels were determined using a portable glucometer. Rats that had become hyperglycemic (blood sugar level > 300 mg/dL) were selected for further experiments.

To evaluate the antihyperglycemic activity of partially purified santalin A, diabetic rats (n=6 per group) were treated intraperitoneally with 0.5 mg/kg (D + S 0.5) or 1 mg/kg (D + S 1.0) body weight for 10 days. Parallelly, control untreated (U), diabetic alone (D), 0.5 mg/kg santalin alone (S0.5) and 1.0 mg/kg santalin alone (S1)

groups were maintained (n=6 in each group). In order to determine the protective effect of santalins on diabetic induction, rats were pretreated orally with either 0.5 mg/kg (S0.5 + D) or 1.0 mg/kg (S1.0 + D) for three days, followed by the administration of alloxan as described above. At the end of each treatment schedule, rats were sacrificed by chloroform asphyxiation and cervical dislocation. The samples of blood and organs (liver, kidneys, and pancreas) were collected from all the rats and stored at -80°C until further analysis.

2.6.1 Lipid Peroxidation Assay

The level of lipid peroxidation (LPO) in the liver, kidneys, and pancreas of rats was estimated as previously described[26]. To one mL of tissue homogenate (10% w/v) of different samples, an equal volume of trichloroacetic acid (TCA) was added and centrifuged at 15,000 rpm. The supernatant was collected, and 0.5 mL of thiobarbituric acid (TBA) was added and kept at 95°C for one hour. The intensity of the color formed was measured at 532 nm, and the levels of lipid peroxidation products were expressed as nanomoles MDA/mg protein. 1,1,3,3-tetraethoxy-propane (TEP) was used as the standard.

2.6.2 Antioxidant Enzyme Assays

The activities of catalase, glutathione peroxidase (GPx), and glutathione S-transferase (GST) were determined as described earlier[27-29]. The tissues taken from different organs *viz*., liver, kidney, and pancreas, were prepared to 10% (w/v) in ice-cold phosphate buffered saline, pH 7.4, and centrifuged at 3000 rpm to remove the debris. The supernatant was used for the determination of the activities of antioxidant enzymes.

2.7 Statistical Analysis

All the experiments were conducted in triplicates, and values were represented as means ± standard deviations of three replicates each. The means of the treatments were statistically analyzed by ANOVA (One-way analysis of variance), and the means were compared at a 5% probability level by Student-Newman–Keul's multiple comparison test using SigmaPlot for Windows (Systat Software, Inc., Bangalore, India). The data obtained was considered to be statistically significant if their *p*-values were less than 0.05 ($p<0.05$).

3. Results and Discussion

The isolation and partial purification of santalin (A) were carried out by column chromatography, preparative TLC, and HPLC. The purified compounds were characterized by LC-MS, HR-MS, and ^{1}H NMR. The santalin A was extracted from the heartwood by sequential treatment with hexane, petroleum ether, chloroform, dichloromethane and methanol. The methanolic filtrate was evaporated to dryness in a rotary evaporator for chromatography using a silica gel column and subjected to gradient elution with increasing polarity of solvents as described in Materials and Methods (Section 2.3). Fractions collected from column chromatography were analyzed in TLC and those that showed red and yellow color bands were subjected to preparative TLC and the compounds corresponding to the red color band were recovered and analyzed by preparative HPLC.

The purified fraction showed a single major peak with a retention time of 17.117 min with 76% purity (Figure 1A) in preparative HPLC. The fraction corresponding to this peak was collected and analyzed by LC-MS. We observed two peaks that were identified as santalin A and santalin B with a molecular weight of 583 g/mol and 597 g/mol, respectively (Figure 1B). The identity of the mixture of compounds represented by the two peaks was also confirmed by LC-MS and HR-MS (Figure 2A to D). The purified santalins were further analyzed by ^{1}H NMR and the major peak observed in ^{1}H NMR corresponded to santalin A (Figure 2E), indicating that this form is more dominant in the purified fraction. Santalin A has the molecular formula -$C_{33}H_{26}O_{10}$, with the molecular structure -6-((3,4-dihydroxyphenyl)methyl)-2,3-dihydroxy-7-(4-hydroxy-2-methoxyphenyl)-9,11 dimethoxy-10H-5-oxatetraphen-10-one. The structural features of santalin A, as determined by ^{1}H NMR, was: H NMR (500 MHz, d6–DMSO): δ 9.77 (s, 1H, OH), 9.61 (s, 1H, OH), 9.51 (s, 1H), 9.28 (s, 1H, OH), 8.62 (s, 1H, OH), 7.06 (s, 1H), 6.98 (d, J = 8.1 Hz, 1H), 6.66 (s, 1H), 6.65 (d, J = 2.2 Hz, 1H), 6.63 (d, J = 1.9 Hz, 1H), 6.56 (dd, J = 8.3, 2.2 Hz, 1H), 6.56 (d, J = 8.2 Hz, 2H), 6.51 (d, J = 8.2, 1.9 Hz, 1H), 6.42 (d, J = 1.1 Hz, 1H), 4.05 (d, J = 14.6 Hz, 2H), 3.89 (s, 3H), 3.89 (d, J = 14.6 Hz, 1H), 3.64 (s, 3H), 3.63 (s, 3H), 3.59 (s, 3H) ppm. The isolation procedure used in the study yielded 4.9 mg partially purified santalin A per gram of heartwood powder.

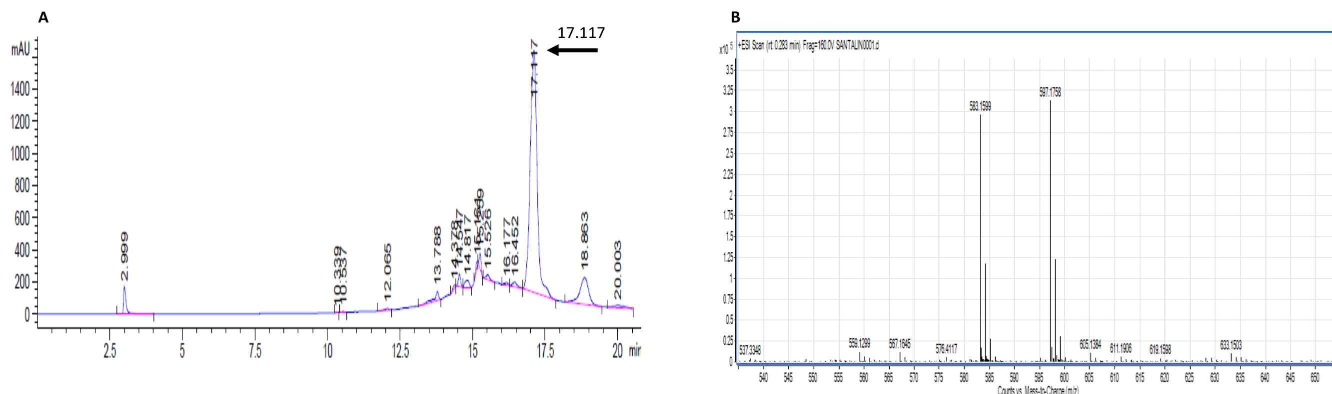
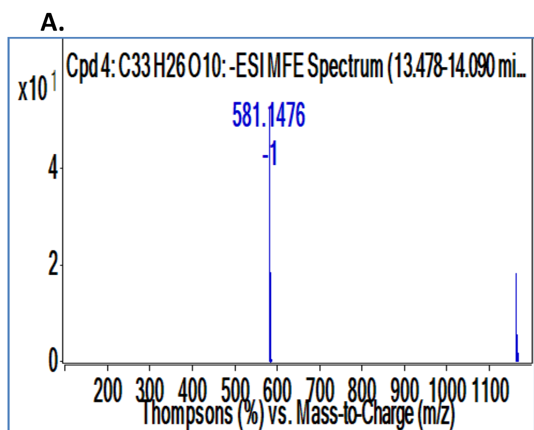
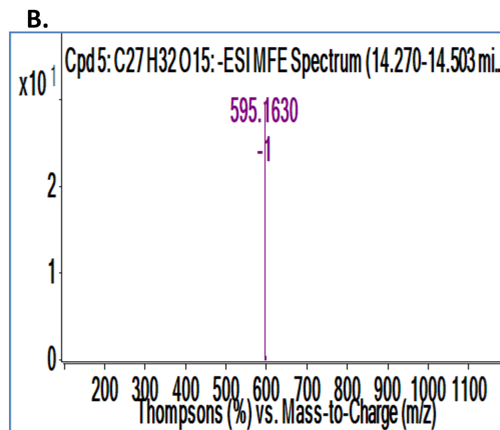
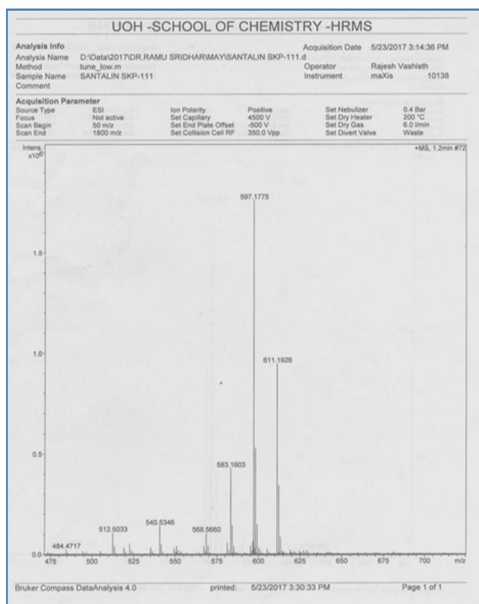
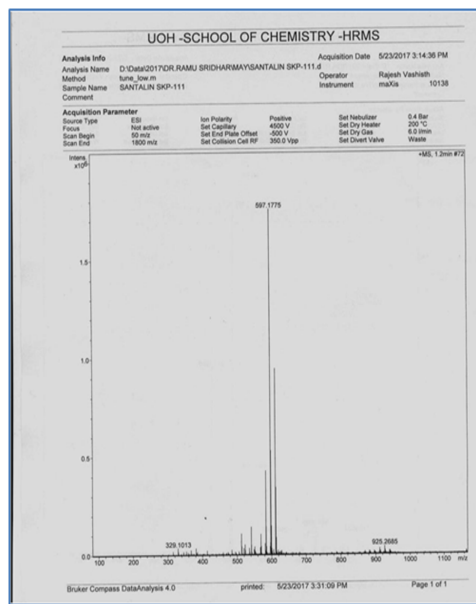


Figure 1. Partial purification of santalin A from the heartwood of *Pterocarpus santalinus*. The methanolic heartwood extract of *P. santalinus* was fractionated by column chromatography and subjected to preparative TLC. The compounds corresponding to red-colored bands in TLC were separated and purified by preparative HPLC. (A) Chromatogram showing peak at 17.117 min corresponds to santalin A. (B) LC-MS spectrograph showing the presence of santalin A and santalin B correspond to the molecular weights 583.1599 and 597.1758, respectively.









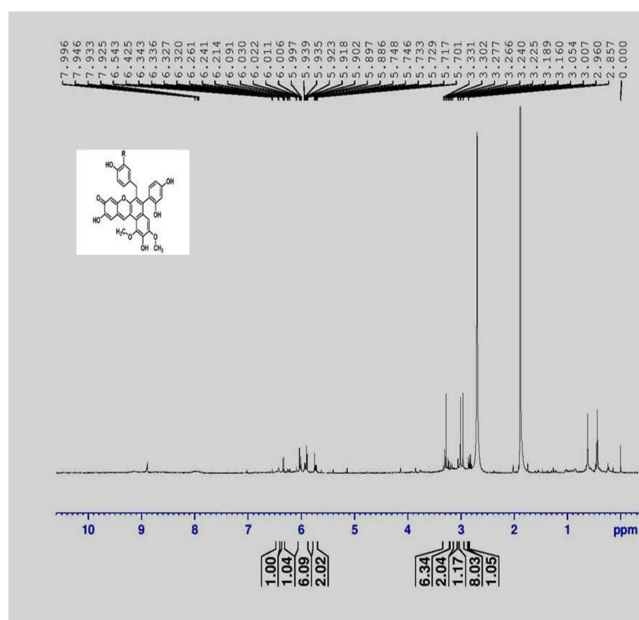


Figure 2. Chemical characterization of santalins. The fraction containing partially purified santalin A was subjected to LC-MS and HR-MS. (A and B) LC-MS spectra of santalin A and B further confirmed their molecular weights. (C and D) HR-MS spectra for santalin A and B. (E) ^{1}H-NMR analysis of the mixture of santalins.

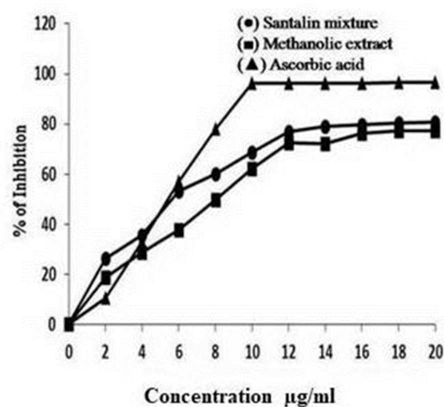
In the last decade, identification of the health benefits of the active principles of medicinally important plants is gaining momentum because of the reason that using the crude plant extracts do not have any control over the concentration of the active principle being administered and on the side effects of the other components. Further, synergy and antagonism exhibited on the active principle by the other components in the plant extracts have been a serious concern to study the actual bioactive properties[30]. Thus, isolation of the active principle or a group of active principles of a similar kind from the plant extracts and testing them for their biological activities represents the best method in ethnopharmacology. In line with this convention, we have partially purified the santalin A from the heartwood of *Pterocarpus santalinus* and assessed its pharmacological properties. Although several researchers have reported the beneficial effects of the extracts of different parts of *P. santalinus*, there have been limited studies describing the pharmacological effects of isolated compounds. The flavonoids, taxifolin, dihydrokaempferol, and naringenin were isolated from the ethyl acetate fraction by centrifugal partition chromatography[31]. 6-Hydroxy-7-methoxycoumarin was isolated from the bark wood of red sanders along with a variety of bioactive molecules[32]. Though the presence of santalins in the heartwood was reported long back, isolation of santalins has not received much attention. Tennakone *et al.* reported a method for santalin isolation from the heartwood of red sanders by the repetition of chromatographic separation[22]. In the present study, this isolation procedure we adopted with slight modifications yielded a partially purified santalin A at a concentration of 4.9 mg per gram of heartwood powder. Preparative HPLC, LC-MS, HR-MS and ^{1}H NMR studies revealed the chemical properties of the santalin A, which agree with the predicted features. The notable point in this study is the isolation of partially purified santalin A using chromatographic separation combined with preparative TLC and HPLC followed by their characterization by LC-MS, HR-MS and ^{1}H NMR analysis. To the best of our knowledge, the aforesaid studies on partial purification of santalin A from the heartwood of red sanders using preparative TLC and HPLC have not been reported so far. Thus, our study contributes to the way to development of the modified isolation protocols for santalin A, although further studies are required to purify santalin A.

The total antioxidant property of partially purified santalin A and the crude heartwood extract was assessed by their ability to reduce Mo (VI) to Mo (V) *in vitro*. The antioxidant capacity of the partially purified santalin A and the crude methanolic extract were found to be 285.55 ± 6.89 and 231.15 ± 11.32 mg AEE/gDW, respectively. The partially purified santalin A demonstrated dose-dependent free radical scavenging ability, as indicated by its ability to inhibit the DPPH radicals (Figure 3A). An inhibition of 80.3% DPPH radicals was observed with an IC_{50} value of 5.49806 μg AAE/mL. The crude methanolic heartwood extract produced free radical scavenging inhibition of 77.41% with an IC_{50} value of 7.78 μg AAE/mL. On the other hand, ascorbic acid brought about the highest inhibition of DPPH by 96.33% with an IC_{50} value of 4.36 μg AAE/mL. The results revealed that the mixture of purified santalin showed higher antioxidant activity than methanolic crude heartwood extract. The use of herbal extracts for medicinal purposes is well known because of the abundant number of bioactive compounds such as flavones, flavonols, flavanones, flavonols, isoflavones, and anthocyanidins therein. The genus *Pterocarpus* is known to be a rich source of flavonoids and related phenolic compounds and hence has been identified as a medicinally important plant with antioxidant properties.

DPPH and TAA methods have been widely used to assess the free radical scavenging activity of phytocompounds due to their sensitivity and rapidity. The water extract of the leaves of *Pterocarpus marsupium* was reported to be exhibiting radical scavenging activity by DPPH reduction[33]. Similarly, the alcoholic extract of the heartwood of *P. marsupium* was shown to scavenge free radicals[34].

There are a few reports concerning the free radical scavenging activity of *P. santalinus*. *In vitro* studies demonstrated the free radical scavenging activity of the methanolic extract of the leaves and heartwood of this plant[19,24,29]. In a rat model, the antioxidant properties of the methanolic extract of bark wood were reported[19]. However, such activity specifically for santalins is not reported yet. Our results based on phosphomolybdenum, and DPPH radical scavenging assays revealed total antioxidant activity and dose-dependent free radical scavenging activity of crude heartwood extract and a partially purified santalin A. Interestingly, the total antioxidant and free radical scavenging activities were more pronounced for partially purified santalin A than the crude extract. To the best of our knowledge, for the first time, we demonstrate the total antioxidant and free radical scavenging activities of partially purified santalin A.

The ability of a partially purified santalin A to control blood glucose levels was assessed in a rat model of alloxan-induced diabetes mellitus (Figure 3B). Administration of alloxan alone resulted in the development of diabetes, which is evident by the elevated levels of blood glucose (270.0±137.0) that are significantly higher than the control group (104.0±8.5 mg/dL) after 10 days. The diabetic rats were then treated with 0.5 (D + S0.5) or 1.0 (D + S1.0) mg/kg body weight of a mixture of santalins. The administration of the partially purified santalin A did not affect hyperglycemia (Figure 3B). The blood glucose levels in D + S0.5 and D + S1.0 groups were found to be 289.0±149.0 and 390.0±185.0 mg/dL, respectively. These results suggest that santalin A might not have hypoglycemic activity. To determine if pretreatment with partially purified santalin A has a protective effect on the onset of diabetes, rats were first treated with 0.5 (S0.5 + D) or 1.0 (S1.0 + D) mg/kg body weight of a partially purified santalin A for three days, following which alloxan was administered to induce diabetes. The development of alloxan-induced diabetes was evident in rats pretreated with 0.5 mg/kg dose, as indicated by higher levels of blood glucose (252.0 ± 126.0 mg/dL). However, the blood glucose levels appeared to be lower than the diabetic control though not significant. Interestingly, blood glucose levels were found to be 120.0 ± 11.0 mg/dL in rats pretreated with 1.0 mg /kg dose of partially purified santalin A (Figure 3B). Treatment with partially purified santalin A alone did not affect blood glucose levels (data not shown).



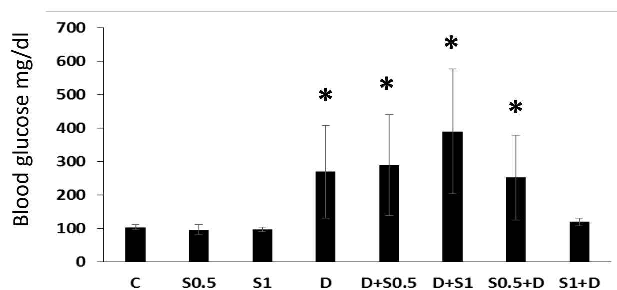


Figure 3. Functional analysis of santalin A. (A) Free radical scavenging activity. The inhibition of DPPH by a mixture of partially purified santalin A, methanolic crude heartwood extract and ascorbic acid was determined by measuring the color intensity of reduced DPPH. (B) Antidiabetic activity of partially purified santalin A in Wistar rats. Alloxan-induced diabetic rats (D) were treated with 0.5 or 1.0 mg/kg body weight (D + S0.5 or D + S1.0)) santalin A. S0.5 + D and S1.0 + D groups indicate that rats were first pre-treated with 0.5 or 1.0 mg/kg body weight of partially purified santalin A for ten days and then administered alloxan. C – untreated control. Values shown are Mean ± S.D. * indicates $p < 0.05$ compared to the control (C) group.

It appears that the partially purified santalin A at a dose of 1.0 mg/kg body weight can prevent alloxan-induced diabetes mellitus. The hypoglycemic activity of extracts of medicinally important plants, including the *Pterocarpus* genus, has been explored extensively to mitigate the side effects of conventionally used drugs. The aqueous extract of *Pterocarpus marsupium* helped in controlled fasting and postprandial blood glucose in rabbits with alloxan-induced diabetes[35]. The anti-hyperglycemic effect of bark wood of *Pterocarpus marsupium* methanolic extract in alloxan-induced diabetes was demonstrated in a rat model[36]. Prevention of hyperinsulinemia and hyperglycemia[37], stimulation of insulin secretion[38], modulation of metabolic alterations[39], and hypoglycemic effects[40-42] of *Pterocarpus marsupium* were demonstrated using different animal models. Because of the hypoglycemic activity, extracts of *Pterocarpus marsupium* were used as an add-on therapy in type 2 diabetic patients[43]. The antidiabetic activity of *P. santalinus* was also demonstrated. The ethanolic, ethylacetate-methanol, and aqueous extracts produced potent hypoglycemic activity in animal models of chemically induced diabetes[21,44-46]. Although hypoglycemic activity potential of the extracts of *P. santalinus* has been shown previously, such an activity with the isolated active principles is not reported so far. In this study, we observed that santalin A did not show any hypoglycemic effect when administered to diabetic rats. However, pretreatment with the partially purified santalin A (S1.0 + D group) prevented the induction of diabetes and the associated hyperglycemia. These results indicate that using santalins as supplement may prevent or delay the onset of diabetes in high-risk individuals. Such a beneficial action hitherto has not been demonstrated and thus assumes enormous clinical significance. Studies in high-risk individuals to evaluate the prevention or delay

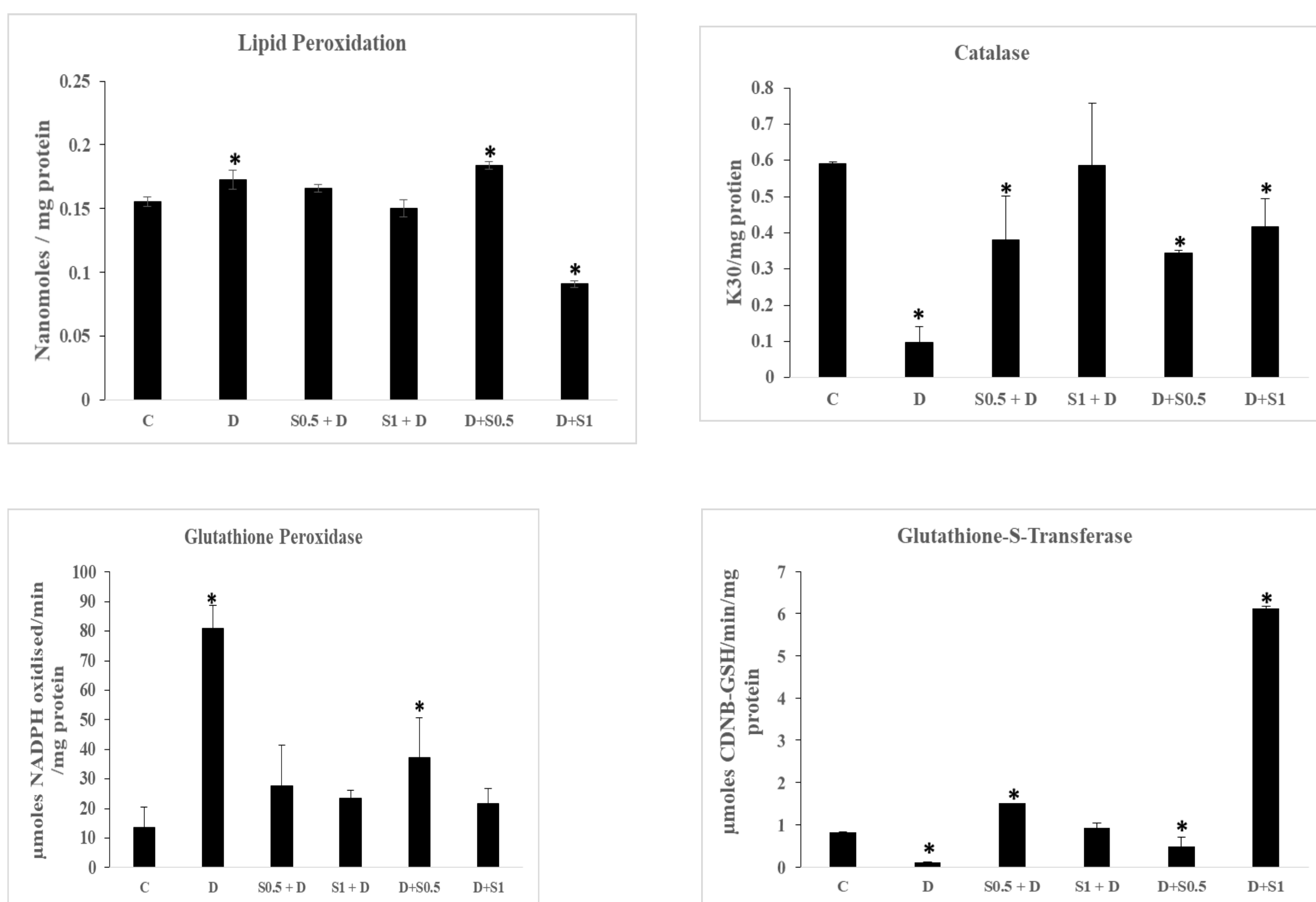


Figure 4. Lipid peroxidation and antioxidant enzyme activities in the liver. Tissue homogenates were prepared from control (C), diabetic (D), santalin pre-treated + alloxan treated (S0.5 + D or S1.0 + D) and diabetic + santalin post-treated rats (D + S0.5 or D + S1.0). (A) Lipid peroxidation and the activities of (B) catalase, (C) glutathione peroxidase, (D) glutathione-S-transferase were determined. Values shown are Mean ± S.D. * indicates $p < 0.05$ compared to the control (C) group.

in onset of diabetes by administration of santalin A are necessary to tap the pharmacological potential.

Alterations in the oxidant and antioxidant status are a hallmark of diabetes. To evaluate the possible intervention of santalin A to mitigate the altered antioxidant activity status, we analyzed the levels of lipid peroxidation and activities of catalase, glutathione peroxidase, and glutathione-S-transferase in the liver, kidney, and pancreas. The levels of lipid peroxides were notably increased in the liver of diabetic rats, and the same was restored to control levels in the S1.0 + D group (Figure 4A). Though a slight reduction was observed in the S0.5 + D group, it was not significantly lower than the diabetic (D) alone group. In the D + S0.5 group, lipid peroxidation remained significantly higher, whereas the same was restored to below control levels in the D + S1.0 group. Catalase and glutathione-S-transferase activities were significantly reduced whereas glutathione peroxidase activity was markedly increased in diabetic rats (Figure 4B-D). Pretreatment with 0.5 or 1.0 mg/kg of a partially purified santalin A resulted in restoration of catalase activity to that of control in the S1.0 + D group and, to a certain extent in the S0.5 + D group (Figure 4B). Restoration of glutathione-S-transferase activity to control levels was observed in the S1.0 +D group, whereas the levels were above control levels in the S0.5 + D and D + S1.0 groups. However, the activity remained significantly lower than control in the D + S0.5 group (Figure 4D). Glutathione peroxidase activity that was significantly increased in the diabetic rats was restored to control levels in diabetic rats that were either administered santalin A either pre- or post-diabetes induction (Figure 4C).

In the kidneys of diabetic rats lipid peroxidation levels were significantly increased, which, however, were lowered in all the treatment groups (Figure 5A), though the decrease in the S0.5 + D group rats was not completely

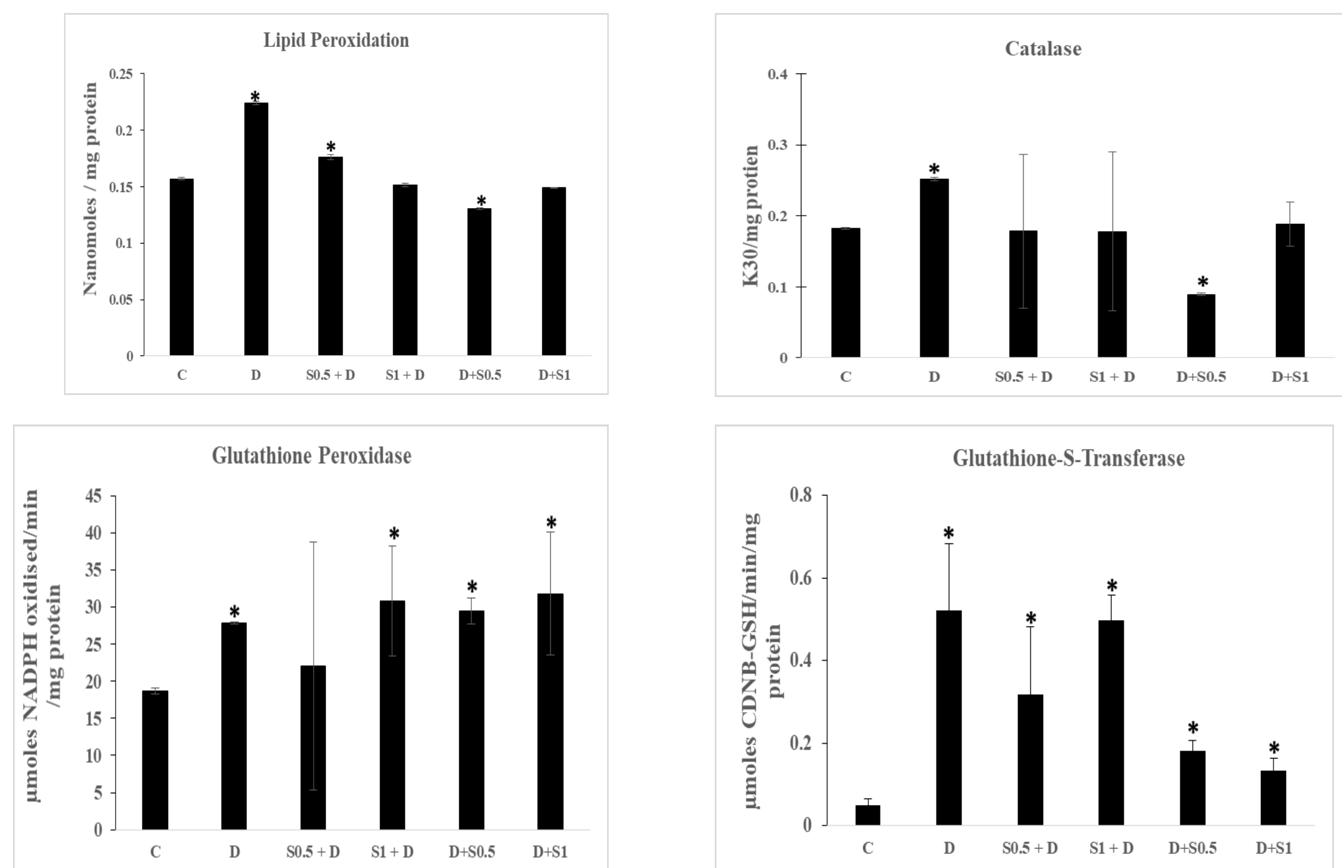


Figure 5. Lipid peroxidation and antioxidant enzyme activities in the kidney. Tissue homogenates were prepared from control (C), diabetic (D), santalin pre-treated + alloxan treated (S0.5+ D or S1.0 + D) and diabetic + santalin post-treated rats (D + S0.5 or D + S1.0). (A) Lipid peroxidation, (B) the activities of catalase, (C) glutathione peroxidase, (D) glutathione-S-transferase were determined. Values shown are Mean ± S.D. * indicates $p < 0.05$ compared to the control (C) group.

restored to the control levels. Compared to the control group, the activities of catalase, glutathione-S-transferase, and glutathione peroxidase were increased in the diabetic rats (Figure 5B-D). Pretreatment with partially purified santalin A, though resulted in restoring the catalase activity to a certain extent, the activities were restored to control levels in the post-santalin (D + S0.5) treatment group (Figure 5B). Glutathione peroxidase activity remained significantly high as in diabetic rats in all the santalin treatment groups (Figure 5C). Glutathione-S-transferase activity in the santalin pretreatment groups remained high, like diabetic rats. In the case of post-treatment groups, the activity was restored only to a certain extent but significantly higher than the control rats (Figure 5D).

In the pancreas, the extent of lipid peroxidation in all santalin treated groups was restored to that of the control group, which was otherwise significantly increased in the diabetic rats (Figure 6A). Catalase activity was significantly decreased in diabetic rats when compared to controls. Pretreatment with partially purified santalin A (0.5 or 1.0 mg/kg) resulted in a significant increase in catalase activity to a level close to control. However, this restoration was not observed in post-santalin treatment groups (Figure 6B). The activities of glutathione peroxidase and glutathione-S-transferase were significantly increased in the diabetic rats (Figure 6C and D). Interestingly, the activity of glutathione peroxidase decreased to below the control levels in all the groups treated with a partially purified santalin A. Glutathione-S-transferase activity was restored to levels in control group in the S0.5 + D, S1.0 + D, and D + S1.0 treatments, except in D + S0.5 group (Figure 6D).

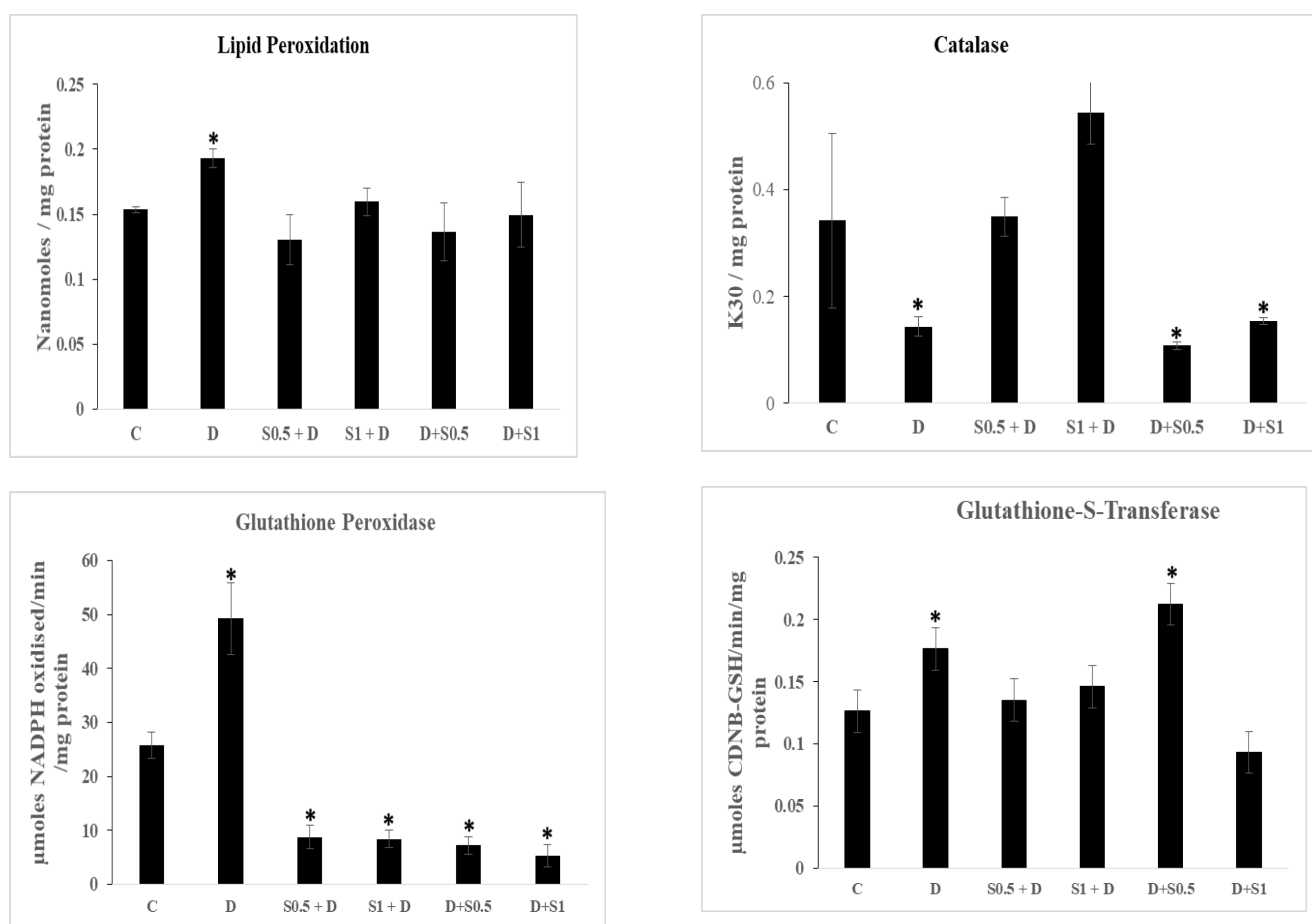


Figure 6. Lipid peroxidation and antioxidant enzyme activities in the pancreas. Tissue homogenates were prepared from control (C), diabetic (D) santalin pre-treated + alloxan treated (S0.5+ D or S1.0 + D) and diabetic + santalin post-treated rats (D + S0.5 or D + S1.0). (A) Lipid peroxidation and the activities of (B) catalase, (C) glutathione peroxidase (D) glutathione-S-transferase were determined. Values shown are Mean ± S.D. * indicates $p < 0.05$ compared to the control (C) group.

Perturbations in the oxidant and antioxidant status are associated with hyperglycemia and diabetes. Increased lipid peroxidation and alterations in the levels of antioxidant enzymes are known to contribute to the pathophysiology of diabetes. Interventions to abrogate or minimize oxidant-antioxidant balance are crucial for the management of diabetic complications, and several bioactive molecules derived from plant extracts have been proposed for such beneficial effects. Antioxidant activity, as indicated by decreased lipid peroxidation, was observed in diabetic rats treated with the methanolic extracts of *P. marsupium*[47-49].

Similarly, the antioxidant potential of the extract of *P. santalinus* was also evaluated. Reduction in the malondialdehyde levels of the brain, liver, and muscle was observed in diabetic rats administered the aqueous extract of the heartwood of *P. santalinus*[44]. Streptozotocin-induced deleterious effects, including lipid peroxidation, were abrogated by the aqueous bark extract of *P. santalinus*[50]. In this study, we observed that pre- or post-treatment with a partially purified santalin A did not cause any significant alteration in lipid peroxidation in the liver, kidney, and pancreas of diabetic rats and were comparable to the control group, except D + S0.5 group wherein lipid peroxidation levels were higher than the control group. Furthermore, the activities of catalase, glutathione peroxidase, and glutathione-S-transferase were found to vary in the liver and kidneys of santalin treated diabetic rats as compared to diabetic rats. On the other hand, the activities of catalase remained higher, whereas glutathione peroxidase and glutathione-S-transferase remained lower in the pancreas of santalin pretreated diabetic rats than diabetic rats. The alterations in oxidant-antioxidant status in santalin pretreated diabetic rats might be primarily because of the prevention of damage to the pancreas and the resultant non-induction of diabetes. Though most of the studies report restoration of antioxidant status in diabetic rats along with hypoglycemic activity by plant extracts, the ability of these compounds to control oxidative stress despite lack of hypoglycemic activity is not documented. The restoration of lipid peroxidation and the activities of antioxidant enzymes in rats that were treated with a partially purified santalin A after diabetes was induced indicate the ability of these compounds to mitigate the complications. Such properties of natural compounds can be exploited to prevent diabetic complications, thereby reducing the morbidity and, lastly to improve the quality of life.

4. Conclusion

In conclusion, we report the isolation, partial purification, and characterization of santalins from the heartwood of *P. santalinus*. The partially purified santalin A exhibited antioxidant and free radical scavenging activities *in vitro*. Administration of partially purified santalin A to diabetic rats failed to reverse hyperglycemia. On the other hand, the development of alloxan-induced diabetes was prevented when rats were pretreated with the isolated santalin A. Our results demonstrate a potential role for santalin A as preventive agents for diabetes in a high-risk population.

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Conflict of interest

The authors declare no conflict of interest.

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