JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2025/47941
RESEARCH ARTICLE

Phytochemical Analysis and *In Vitro* Antidiabetic Assessment of *Artocarpus hirsutus* Leaves

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Abstract

Background: Diabetes a chronic endocrine disorder affects mainly glucose metabolism which is characterized by increased blood glucose and glucose intolerance, caused by decreased insulin production or insulin ineffectiveness, or a combination of both. This becomes a global health threat, and International Diabetes Federation reported that, in 2021, 537 million people between 20 to 79 ages will be suffering with Diabetes. Artocarpin, b -sitosterol, b cycloartocarpin, cudraflavone A and C, and oxyresveratrol are some of the biologically important phytomolecules isolated from *Artocarpus* species. **Aim:** This study focussed on to the antioxidant and antidiabetic activity of *Artocarpus hirsutus* leaves. **Methods:** The sequential solvent extraction with increasing polarity was achieved by using hexane, chloroform, ethanol, and water. *In vitro* analysis such as DPPH, nitric oxide scavenging, lipid peroxidation and alpha-amylase assays are carried to check out pharmacological activity. **Results:** After completion of the *in vitro* assays, the selected ethanolic extract was subjected to phytochemical separation and isolation by utilising column chromatography. Four distinctive fractions were isolated using column chromatography and *in vitro* assays performed to measure antioxidant and antidiabetic activity. Fraction IV isolated from ethanolic extract exhibited highest antioxidant activity DPPH (IC_{50} of 145.2 ± 0.04µg/mL), and nitrous oxide scavenging property (IC_{50} of 83.95 ± 0.05µg/mL) when compared to standard. Fraction IV showed effective lipid peroxidation action, in a dose-dependent manner when compared to control perilla oil. Fraction IV contains stronger alpha-amylase activity (IC_{50} 0.068 ± 0.1414µg/mL), when compared ascorbic acid. **Major Findings:** Fraction IV isolated from the ethanolic extract exhibits a stronger antioxidant property and alpha-amylase inhibition properties. Additional studies are suggested to identify and define the chemical components and its mechanism responsible for the above effects.

Keywords: *A. hirsutus*, Alpha-amylase Inhibition, Antioxidant, Column Chromatography, Phytochemical Isolation

1. Introduction

Diabetes a chronic endocrine disorder affects mainly glucose metabolism which is characterized by increased blood glucose and glucose intolerance, caused by decreased insulin production or insulin ineffectiveness, or a combination of both. This becomes a global health threat, and International Diabetes Federation reported that, in 2021, 537 million people between 20 to 79 ages will be suffering with diabetes. The frequency of this disease is expected to increase drastically, with projections tend to shoot up to 643 million by 2030 and may attain 783 million by 2045[1,2]. Owing to higher side effects and cost the utilization of plant based medication tends to rise in recent years. In response to this alarm increasing, the shift in the usage of plant medicines has grown in recent years among the population for both prevention and treatment. The active natural compounds from plants have been studied for their therapeutic efficacy and are considered as safer than the currently available medications. Traditional medicine across various countries has a higher value for their therapeutic property, and estimated that at least four billion peoples in emerging nations might rely on plants based medications for treating diabetes. Scientific studies have continuously insisted that medicinal plants, along

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Article Received on: 27.12.2024 Revised on: 06.05.2025 Accepted on: 21.05.2025

with nutraceuticals and essential elements, may play a major role in managing diabetes[3,4]. Ethnobotanical survey suggests that around 1,200 uncommon plants across the globe might possess significant anti-diabetic activities.

Chalcone, artocarmitin B, artocarpin, cudraflavone C, albanina, brosimone I, norartocarpanone, euchrenone, Moracin M and C, albafuran B are the phytomolecules isolated from *Artocarpus* species[5]. Artocarpin was successfully isolated from the root wood of *Artocarpus kemando* Miq, proved for antidiabetic and antibacterial activity[6]. The secondary plant metabolites identified from *Artocarpus* species may contribute to various pharmacological activities. They perceive a lower side effects and comprehensive pharmacological activities which make medicinal plants an attractive source of drug discovery for safer hypoglycemic agents. *A. hirsutus*, (commonly known as wild jack), a member of *Moraceae* (mulberry) family, consists of around 60 trees and shrubs holds a considerable biological activity. The various parts of *A. hirsutus* have been traditionally used to treat skin diseases, intrinsic haemorrhages, and poisons. The bark powder is used to treat sores; dried leaves are applied to buboes and hydrocele. *Artocarpus hirsutus* leaves have been used locally in managing diabetes mellitus with sparse scientific information. Due to the presence of bioactive phytoconstituents especially flavanoids, polyphenols and terpenoids *A. hirsutus* plant leaves was chosen for this study. Added this plant was generally considered as safe with fewer side effects. With the traditional utilization and therapeutic value, this study lined up to extract, isolate phytomoieties from of *A. hirsutus* leaves and appraise their antidiabetic activities[7].

In the absence of systematic reports in the literature, this study augment the evidence for reinforce the use of medicinal plants in control of diabetes, focussing on the pharmacological eminence of *A. hirsutus* as anti-diabetic compounds.

2. Materials and Methods

The following chemicals and reagents were used in this study hexane, chloroform, ethanol, Dragendorff's reagent, hydrochloric acid, lead acetate, sulfuric acid, sodium carbonate, ferric chloride, n-hexane, ethyl acetate, acetic acid, and silica were all procured from Molychem (Global Scientific Company, Erode). Mayer's reagent and sodium hydroxide were obtained from Isochem (Chemico Glass and Scientific Company, Erode), while Hager's reagent was sourced from Ozone and Molisch reagent was supplied by Spectrum. Furthermore, sodium nitroprusside and soluble starch were obtained from Fisher Scientific, while gallic acid was sourced from SRL. Alpha-amylase (HIMEDIA) was also used in the biochemical analyses.

2.1 Extraction

The *A. hirsutus* Lam leaves were collected in the month of January from Munnar district, Kerala, and authentified by Dr. M. U. Sharief, Scientist 'F' and Head of Office, Botanical Survey of India, Coimbatore. The leaves were cleaned and allowed to dry before being ground into a coarse powder. A specific quantity of the powder, weighing 1000 gms, was subjected to successive solvent extraction using maceration process. These solvents, selected based on their ascending polarities, included hexane, chloroform, ethanol, and water. For the extraction process, firstly hexane was combined with the powdered material and the mixture was stirred at 500 revolutions per minute (rpm) and maintained at 40° C for 2 hours. Then the mixture was vacuum filtered and the marc was separated. This procedure was iteratively performed using the subsequent solvents under the same operational conditions to achieve successive extraction[8].

2.2 Preliminary Phytochemical Analysis

Qualitative phytochemicals screening of of *A. hirsutus* leaf extracts was done to detect the presence of various phytochemicals like phenols, saponins, tannins, flavonoids, alkaloids, steroids and glycosides using the standards method as described[9,10].

2.3 Biochemical Analysis

2.3.1 DPPH Assay

2, 2-Diphenyl-1-picrylhydrazyl assay was used to measure the antioxidant activity. 5µl of sample was mixed with varying concentrations, with 0.1ml of DPPH solution in a 96-well plate. The process was repeated 3 times. Each time, blank wells were made up with 5µl of the extracts at different concentrations and

0.2ml of DMSO/Methanol. The control group consisted of untreated wells. For half an hour, the plate was left to incubate in darkness. After incubation, the absorbance at 517 nm measured using microplate reader (iMark, Bio-Rad). The control was the reaction mixture that included 20µl of deionized water. Inhibition percentage as a percentage of control was used to indicate antioxidant activity. We used GraphPad prism 6 to get the IC_{50} values[11-13].

% inhibition= $(Ab_{Con}/Ab_{sam}) \times 100$

2.3.2 Nitric Oxide Radical Scavenging Assay

The assay medium was prepared using 40µL of deionized water, 50 µL of 10 mM sodium nitroprusside, 10 µL of the sample or the standard gallic acid, or a blank to determine the NO radical scavenging activity. The control group received the reaction mixture that had not been treated in any way. For 15 minutes, this combination was pre-incubated at room temperature with light. The test and control wells were each treated with 100µL of Griess reagent after incubation. The plate was incubated for 10 minutes at room temperature for stabilized chromophore formation. The microplate reader (iMark, Bio-Rad) was used to estimate the absorbance at 540 and 660 nm. GraphPad prism 6 software is used to get the IC_{50} values[14,15].

2.3.3 Lipid Peroxidation

Mixing raw perilla oil with different quantities of extracts and water creates an oil emulsion, which is used to examine lipid peroxidation. Following that, 10% gum Arabic was added to the raw perilla oil to serve as an emulsifier. The mixture was then homogenized for 16 minutes. Finally, the oil emulsion was subjected to lipid oxidation at 40° C in an incubator. After incubation for 10 days, the perilla oil emulsions were collected. The TBARS assay used to evaluate lipid peroxidation using raw perilla oil. To the eppendorf tube 40 µL of a 20 mM phosphate buffer (pH 7.0, 10 µL), sample at various concentration, 200 µL of 0.1 N hydrochloric acid, 100 µL of 0.7% 2-thiobarbituric acid, 30 µL of 10% phosphotungstic acid, and 50 µL of 3% sodium dodecyl sulphate is added, tightly sealed and heated to 100° C for 30 minutes. Then 400 µL of n-butanol was added to the reaction mixture and centrifuged at 3000 rpm for 10 minutes. The supernatant collected was loaded in a 96-well plate and the fluorescence intensity is measured at 515 nm and 555 nm[16,17].

Malondialdehyde concentration (M) = (Abs 532nm/$1.56x10^5$) × Dilution Factor

2.3.4 Alpha Amylase Activity

The GOD-POD technique was used to measure the alpha-amylase activity. 10 µl of an enzyme solution containing, 10 µl of sample and 50 µl of soluble starch was added in 96-well plate. Then the mixture was incubated for fifteen minutes. 100 µl of GOD-POD Reagent was added to each well, and incubated at room temperature for 10 minutes and the absorbance measured at 490 nm. Acarbose acts as the positive control. GraphPad prism 6 was used to get the IC_{50} value[18-22].

2.4 TLC Analysis

Based on the yield, presence of phytochemical constituents in extracts and *in vitro* tests the extract with maximum activity will be chosen for TLC analysis. Since Thin Layer Chromatography (TLC) is rapid and its elution patterns are usually carried over to column chromatography, the selected extract was subjected to one-dimensional TLC to achieve the best solvent system. For the TLC process, Silica gel 60 will be used as the stationary phase. Different solvents in various ratios were used as the mobile phase. The distances travelled by each compound will be compared with the solvent front, and the Retention factor (R_f) values will be ascertained[8,23].

2.5 Liquid-liquid Extraction

The selected extract will be subjected to separation using a separating funnel using the optimized solvent ratios chosen above. The fractions collected from each extraction were pooled and then dried for further analysis[24].

2.6 Column Chromatography

The separated compound will be subjected to column chromatography on 45 × 4.5 cm open column containing silica gel. Using gradient elution technique, the content of column is dissolved using a mobile phase solvent solution. The fractions separated based

on TLC profiles will be collected and used for further biochemical assays[24].

2.7 Biochemical Assay of the Isolates

The fractions isolated by silica gel column chromatography were subjected for antioxidant and antidiabetic activity by afore mentioned procedure.

3. Results and Discussion

3.1 Extraction

The selection of solvents for phytochemical extraction is found to affect the resulting extract volume and its bioactivity. The utilisation varying polarity solvents can render a better mass medium transfer, along with extensive coverage of extraction efficiency. The present work is the first trial to report the sequential solvent extraction of *A. hirsutus* leaves employing hexane, chloroform, ethanol and water to study its antioxidant and antidiabetic properties. The obtained yields of each extracts are presented in Table 1.

Table 1. Percentage yield of the different extracts

Sample	Yield (g)	Percentage Yield (%)
Hexane	53.04	1.06%
Chloroform	26.19	0.52%
Ethanol	69.48	1.39%
Water	24.36	0.49%

The highest yield was obtained with ethanol, followed by hexane, chloroform, and water. The ethanol extraction produced a yield of 1.39 %, suggesting its superior efficacy in extracting polar compounds. The hexane extraction, aiming non-polar components, resulted in 1.06 % yield representing the presence of non-polar constituents. Low yields of 0.52 % and 0.49 % were obtained by chloroform and water extractions respectively.

3.2 Preliminary Phytochemical Analysis

The results of qualitative phytochemical screening are presented in Table 2. The phenols, flavonoids and tannins found in the extracts are of interest since it is reported in literature that these compounds are responsible for the biological activity[25].

Table 2. Preliminary phytochemical analysis

Phyto-chemical	Extracts			
	Hexane	Chloro-form	Ethanol	Aque-ous
Phenols	-	+	+	+
Flavonoids	+	+	+	+
Tannins	-	-	+	+
Saponins	-	-	+	+
Terpenoids	-	+	+	+
Alkaloids	+	-	+	-
Proteins	-	-	-	-
Carbohy-drates	-	-	+	+
Glycosides	-	-	-	-

3.3 Biochemical Analysis

3.3.1 DPPH Assay

Antioxidant activity is influenced by various factors, and a single antioxidant property model cannot extensively represent the antioxidant capacity of all samples. Hence, different antioxidant activity assays are employed to investigate the versatile mechanisms of antioxidant action. Free radicals play a pivotal role in biological damage and aging process. DPPH a purple colored stable free radical turns into yellow compound on reaction with an antioxidant which is determined by a decrease in its absorbance at 517 nm. The extent of this reaction depends on the hydrogen ion donating ability of antioxidants; hence this assay is used commonly to measure the free radical scavenging potential of herbal extracts[26,27].

From the outcomes of the DPPH assay the crude ethanol extract (IC_{50} value of 2752 ± 24µg/mL) showed a comparable activity with respect to ascorbic acid (IC_{50} 0.8268 ± 0.31µg/mL). When compared between the extracts the ethanol extract (IC_{50} value of 2752 ± 24µg/mL) showed a sharp free radical scavenging activity followed by aqueous extract, chloroform extract, and hexane extracts (Figure 1). We can infer that *A .hirsutus* extracts of high polarity solvents were considerably more effective free radical scavengers than those of less polar solvents. The DPPH scavenging activity of *A. hirsutus* may be due to the presence of flavonoids and other polyphenols. In addition the difference of IC_{50}

between extracts might be due to variation in bioactive composition of analyzed extracts[28,29].

3.3.2 Nitric Oxide Radical Scavenging Assay

Nitric oxide is an influential signalling compound in numerous biological processes, including vasodilation, neurotransmission, regulation of hormone secretion, killing of micro organisms and destruction of tumour cells. Nevertheless overproduction of NO can impose undesirable biological effects due to its pro-oxidant activity at higher concentrations and has ability to generate extremely reactive peroxynitrites that cause damage to biomolecules. Thus NO scavenging evaluation of plant extracts is frequently employed to carry out the antioxidant potential[30]. The ethanolic crude extract (IC_{50} value of 205.4 ± 0.019µg/ml) exhibit a robust NO scavenging with respect to gallic acid with IC_{50} value of 16.96 ± 0.036µg/ml. The excess NO production is associated with insulin resistance and the ethanolic extract may tend to decrease the oxidative stress leading to improved insulin sensitivity (Figure 2)[31,32].

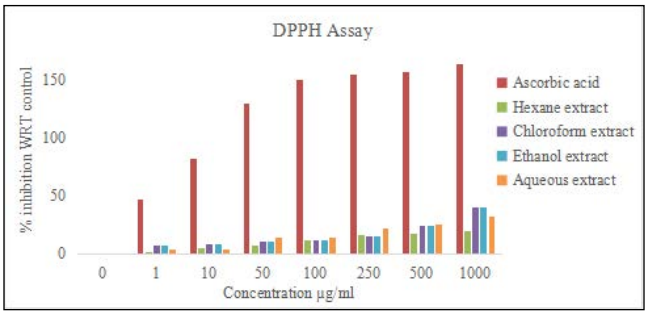


Figure 1. DPPH radical scavenging activity.

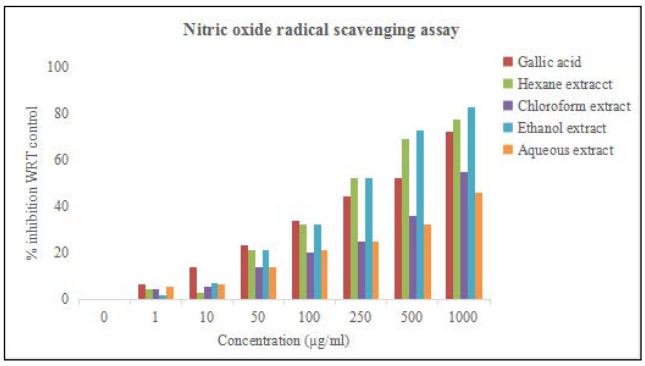


Figure 2. Nitric oxide radical scavenging assay.

3.3.3 Lipid Peroxidation

Higher oxidative stress indicated by increased lipid peroxidation plays an important role in Diabetic progression, can lead to insulin resistance, dysfunction of β-cell and increased diabetic complications[32]. The lipid peroxidation assay was performed to measure the decreased lipid peroxidation levels brought by extracts at varying concentrations. The measurement of Malondialdehyde (MDA) levels is a crucial for lipid peroxidation where decreased MDA is an indication of lesser the lipid peroxidation and increased antioxidant activity[33-35]. The ethanol extract exhibited the most substantial decrease in MDA content demonstrating its potent antioxidant activity when compared to control (Perilla oil) which showed a constant MDA levels across all concentrations. The hexane extract showed a moderate reduction followed by chloroform and aqueous extract (Figure 3).

3.3.4 Alpha Amylase Inhibition Assay

One of the most important reasons for postprandial hyperglycemia are amylases a group of enzymes that act on carbohydrate; mediate the breakdown of dietary starch to glucose[36,37]. Amylase inhibition can delay the glucose overproduction and decrease glucose overproduction[38,39]. Lowered absorbance indicates a stronger alpha-amylase inhibition activity of the extracts.

The ethanol extract showed a potential α-amylase inhibition with IC_{50} value of 0.3364 µg/ml, which can be compared to acarbose, an alpha-amylase inhibitor with IC_{50} value of 0.2228 µg/ml (Figure 4). The proposed mechanism for above amylase inhibitory action may

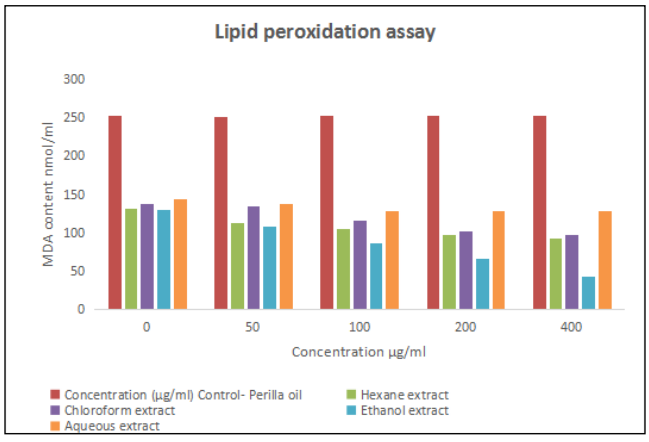


Figure 3. Lipid peroxidation Assay.

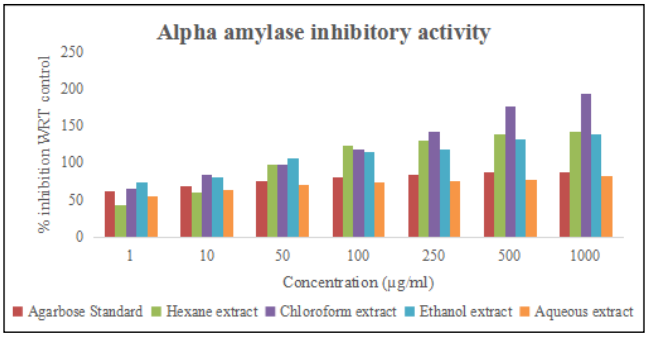


Figure 4. Alpha amylase inhibition assay.

be due to the formation of covalent bonds between polyphenols and the active site of enzymes, and change in microstructure of starch by non-covalent binding between polyphenols and starch[40].

The exact antioxidant and antidiabetic mechanism of *A. hirsutus* leaf extracts was unclear. However, the preliminary phytochemical analysis of extracts shows that the ethanolic leaf extract indicates the presence of phenols, flavanoids and terpenoids which may augment for the free-radical scavenging. The enzyme alpha-amylase found in the salivary, intestinal mucosal and pancreatic secretions, involve in the breaking the α-1-4-glycosidic bonds in polysaccharides thus increasing the blood glucose[41]. Antidiabetics are agents that should be capable to reduce blood glucose or increase the insulin sensitivity. The current *in vitro* studies demonstrate an appreciable antioxidant and α-amylase inhibitory activity of various *A. hirsutus* leaf extract. Based on result outcomes from preliminary phytochemical analysis, *in vitro* antioxidant and antidiabetic assay, the ethanolic extract was chosen and subjected to further studies.

3.4 TLC Analysis

The TLC analysis was performed using different solvent systems. The analysis showed the presence of multiple constituents in the extract indicated by the presence of unique R_f values. Four distinct spots (R_f 0.39, 0.48, 0.64, and 0.86) were developed using toluene and ethyl acetate in the ratio of 9.9: 0.1 using iodine chamber for plate development. This choice was made based on the clear and well-defined compound separation on the TLC plate, which suggests strongly that this solvent combination is optimal for isolation of various secondary metabolites present in the ethanol extract[42].

3.5 Column Chromatography

The ethanolic extract was subjected to liquid-liquid extraction using ethylacetate and water. The phytochemical in the ethyl acetate fraction were further fractionated using silica gel column chromatography by gradient elution technique[43,44]. Four distinct fractions were separated successfully with red colour 1st fraction (2 mg), brown colour 2nd fraction (1.31 mg), greenish yellow 3rd fraction (1.02 mg) and white coloured 4th fraction (4.2 mg) respectively[45].

3.6 Biochemical Assay of the Isolates

3.6.1 DPPH Assay

The DPPH assay quantifies the antioxidant competence of separated fractions, with Fraction IV (IC_{50} 145.2 ± 0.04 µg/ml) indicating highest antioxidant activity in contrast to that of ascorbic acid, (IC_{50} 8.541 ± 0.02 µg/ml) (Figure 5). The bleaching of DPPH absorption is an indication of the ability of tested fractions to scavenge free radicals[28,29]. The low IC_{50} value of fraction IV may be due to presence of pharmacologically active bioactive compound.

3.6.2 Nitric Oxide Radical Scavenging Assay

In the nitric oxide scavenging assay among the isolated fractions, Fraction IV displayed a potential activity (IC_{50} 83.95 ± 0.05 µg/ml) when compared to the standard, gallic acid (IC_{50} 16.79 ± 0.043 µg/ml) (Figure 6). Hence the isolated fraction IV may contain active

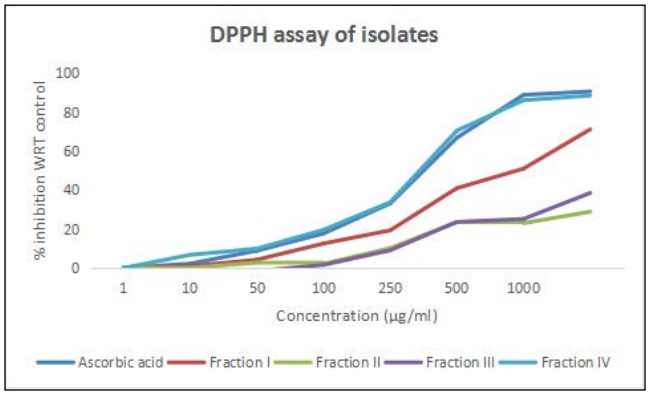


Figure 5. DPPH assay of standard and the isolated fractions.

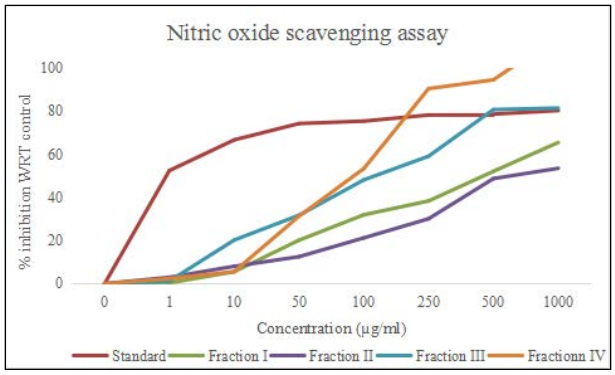


Figure 6. NO scavenging assay of standard and the isolated fractions.

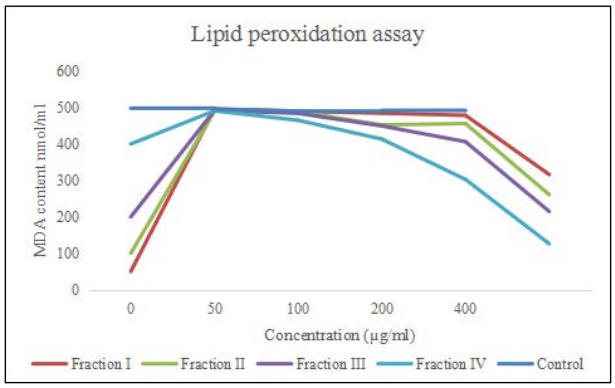


Figure 7. Lipid peroxidation-Fraction I, Fraction II, Fraction III, Fraction IV.

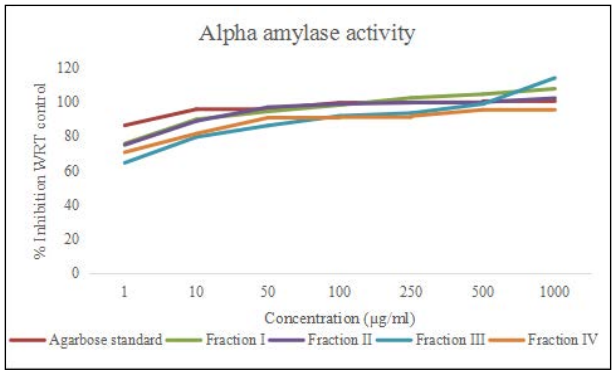


Figure 8. Alpha amylase activity of the standard and the isolated fractions.

phytoconstituent that may effectively scavenge nitric oxide as that of standard[31,35].

3.6.3 Lipid Peroxidation Assay

In the lipid peroxidation assay the control results indicate consistent value across all concentrations. As the concentration of the fractions increased, all fractions exhibited a reduction in MDA content, reflecting their ability to inhibit lipid peroxidation. Fraction IV showed the most significant reduction in MDA content when compared to control Perilla oil. This notable decrease suggests that Fraction IV has the strongest antioxidant potential among the isolated fractions (Figure 7)[33-35].

3.6.4 Alpha Amylase Activity

In alpha-amylase inhibition activity of the 4 isolated fractions the Fraction IV exhibited the highest inhibitory activity (IC_{50} 0.068 ± 0.1414 µg/ml) when compared to acarbose, (IC_{50} 0.222 ± 0.11 µg/ml). This suggests that Fraction IV contains highly effective compounds for inhibiting alpha-amylase, making it the most promising fraction in this assay (Figures 8). This reduction in IC_{50} of Fraction IV in comparison to standard may be due to presence of highly active antidiabetic compound in the separated fraction[36-38].

4. Conclusion

The present study systematically evaluates the antioxidant and antidiabetic potential of *A. hirsutus* leaves. All the 4 extracts *viz* hexane, chloroform, ethanol, and water were subjected to *in vitro* DPPH, nitric oxide scavenging, lipid peroxidation, and alpha-amylase inhibitory activities. The ethanol extract with remarkable bioactivity in the entire assay was chosen and subjected to further fractionation *via* column chromatography. The chromatographic separation yielded 4 distinct fractions, with Fraction IV demonstrating the most potent biological activities across all biochemical assays. The fraction IV exhibited strong antioxidant properties, effective nitric oxide scavenging, substantial lipid peroxidation inhibition, and the highest alpha-amylase inhibitory activity. Overall, this study confirms that *A. hirsutus* leaves are a promising source of bioactive chemicals with significant antidiabetic potential. The comprehensive analysis of unique solvent extraction methods and subsequent fractionation highlights the effectiveness of systematic extraction and chromatographic techniques in identifying and isolating compounds with strong bioactivity. The promising results from Fraction IV warrant further investigation, potentially leading to the

development of natural therapeutic agents for diabetes management.

5. Author Contribution

Rajeswari Rajamani- Conceptualization, methodology, investigation, writing original draft. Kumudha Dhamotharasamy- Formal analysis, editing, visualization and supervision. All authors have read and agreed to the published version of the manuscript.

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