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Cow Urine Mediated Poly Herbal Formulation Preparation, Characterization, and Assessment of Antiglycation and Anti-Diabetic Activity

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Abstract:- present study has the objective of preparing, characterizing, and evaluating the antiglycation and antidiabetic activity of polyherbal formulations mediated by cow urine. The formulations were prepared using various herbs, and their phytochemical constituents were characterized using standard methods[1]. The formulations' ability to prevent the formation of advanced glycation end products (AGEs) was assessed by evaluating their antiglycation activity.[2,3], The antidiabetic activity was assessed by measuring their ability to inhibit α-amylase and α-glucosidase enzymes. The findings revealed that the polyherbal formulations formulated with cow urine exhibited notable antiglycation and antidiabetic properties compared to the control groups. The study concludes that polyherbal formulations mediated by cow urine hold potential therapeutic applications in the management and treatment of diabetes. [1].

Keywords:- *RSA, OS, IC50, AGEs, O.D.*

I. INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder distinguished by persistent elevated levels of blood sugar.[5], affecting an estimated 9.3 percent of adults in India, making it the country with the highest number of diabetes patients. The global incidence of diabetes is also increasing, with estimates suggesting that by 2025, the number of cases in India could rise to 57.2 million. Diabetes can lead to severe short-term and long-term complications, including heart disease, brain damage, and the necessity for amputations, thereby posing a significant threat to life. Oxidative stress is also implicated in diabetic complications, making the identification of appropriate antidiabetic and antioxidant treatments vital [4]. One possible approach is inhibiting the enzymes involved in the initial stages of type II diabetes, which can significantly reduce postprandial blood glucose levels. The non-enzymatic glycation reaction between sugars and proteins contributes to complications in diabetes by forming advanced glycation end products (AGEs), leading to micro and macrovascular complications. Exploring compounds with AGEs inhibitor activity could present a promising therapeutic strategy for preventing diabetes and its associated pathogenic complications [2]. Different treatments are used to control diabetes, including insulin therapy, pharmacotherapy, and diet therapy. [6]

Table 1 Acronyms

Acronym	Full Form	Acronym	Full Form	Acronym	Full Form
RSA	Radical scavenging activity	SD	Standard deviation	ml	Mili liter
TPC	Total Phenol Content	Std.	Standard	µl	Micro liter
NA	Not Applicable	O.D.	Optical density	M	Molar
RNS	Reactive nitrogen species	g	Gram	W	Weight
IC	Inhibition concentration	mg	Mili gram	V	Volume
nm	Nano meter	µg	Micro gram	mM	Mili mol
°C	Degree Celsius	dl	Deci liter	Conc.	Concentration
D/W	Distilled water	Hb	Hemoglobin	DPPH	2,2-diphenyl 1-picrihydrazyl
IC50	Inhibition concentration	DMSO	Dimethyl sulphoxide	NO	Nitric oxide
PBS	Phosphat buffer saline	NBT	Nitro-blue tetrazolium	DM	Diabetes mellitus
BSA	Bovine serum albumin	SNP	Sodium nitroprusside	IDF	International diabetes federation
RPM	Rotation per minute	OS	Oxidative stress	ADA	American diabetic association
AGEs	Advanced glycation end product	ROS	Reactive oxygen species	WHO	World health organization
FCR	Folin - ciocaltear reagent	NaCl	Sodium chloride	NaOH	Sodium hydroxide

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II. MATERIAL

➢ *Plant samples were collected from various locations from Agricultural University, Anand, Gujarat, and Mesarsh Mohanlal manual shell shop, Anand Also Cow Urine was collected from Shri Laxminarayandev Gaushala, Vadtal Plant, and Cow Selection was based upon morphologically normal in the sense of not being infested by diseases.*

➢ *Sample Preparation: -*

The fresh leaves of plant materials were washed completely and then kept for shade-drying it about 8 to 10 days with frequent shifting. The dried materials were then kept in a hot air oven at 45-50º C for about an hour and after that, it was ground using a mortar and pastel to form a fine powder which was then further subjected to extraction by Methanol using soxhlet. About 3 grams of the pulverized samples were dissolved in 150 ml of methanol, and then the extract was filtered using Whatman filter paper no. 1. Following that, the extract underwent evaporation in a rotary evaporator, and the residues were dissolved in dimethyl sulfoxide (DMSO), which were subsequently stored in the refrigerator at 4°C for further analysis. [7]Costusigneus(Insulin), [8]Also, plant samples were extracted by Fresh cow urine distillation. And then Ark was stored in a dark airtight bottle at room temperature [1]

➢ *Determination of Plant Extract, Ark Yield, and (%) Yield Calculation:-*

The yield of the extract obtained was formulated using yield (%) = (weight of extract without solvent/weight of the sample taken) × 100)[7], Costusigneus(Insulin), [8]

➢ *Qualitative Phytochemical Analysis:-*

Methanolic extract of Withaniacoagulans (Pannerpushpam), Holarrhena Antidysenterica(Indrajav)[7] Costusigneus(Insulin), [8]. Also, plant samples were extracted by Fresh cow urine distillation [1] and then subjected to primary phytochemical quantitative screening for the detection of various plant elements.

➢ *Test for Alkaloids:*

- *Wagner's Test:*

A portion of the extract was treated with Wagner's test reagent, and the development of a reddish-brown color precipitate was observed.

➢ *Test for Flavonoids:*

- *Lead Acetate Test:*

A portion of the extract was reacted with lead acetate, resulting in the formation of white precipitation.

➢ *Test for Tannins:*

- *Braymer's test:*

A small amount of the extract was treated with 10% alcoholic FeCl3 solution, resulting in the formation of a bluish or greenish-colored solution.

➢ *Test for Saponins:*

- *Foam test:*

A few samples were stirred with water and showed that the formation of foam persists.

➢ *Test for Carbohydrates:*

- *Molisch's Test:*

A few drops of Molisch's reagent were added to the extract, followed by the careful addition of 1 ml of concentrated H_2SO_4 at a 45-degree angle to the test tube. The reaction mixture was allowed to stand for approximately 2-3 minutes before diluting it with 5 ml of distilled water. The appearance of a dull violet color ring at the interphase of the two layers indicated a positive test result.

- *Benedict's Test:*

To each extract, 0.5 ml of Benedict's reagent was added, and the mixture was then incubated for 2 minutes. The appearance of a green, yellow, or red color indicated the presence of reducing sugars.

➢ *Test for Sterols:*

- *Liebermann-Burchard Test:*

Extracted fraction underwent ethanol and H2SO4 treatment, and the development of purple or green color was monitored.

➢ *Test for Phenols:*

- *Test for Ferric Chloride:*

A small amount of the extract was mixed with a 5% solution of ferric chloride, and the development of a blue color or black hue was observed.

➢ *Test for Anthocyanin:*

- *NaOH Test:*

1 ml of the extract was treated with 2M NaOH, and the formation of a blue to green color was observed.

- *Biuret Test:*

2 ml of filtrate was added to 1 drop of 2% copper sulfate solution and ethanol (95%), followed by an excess of potassium hydroxide pellets. The presence of proteins was indicated by the appearance of a pink color.

- *Ninhydrin Test (amino acids):*

The extract was treated with aqueous Ninhydrin, and the presence of amino acids was indicated by the appearance of a purple color.

➢ *Determination of Total Phenol Content:*

The determination of total phenolic content (TPC) followed a standardized protocol. Approximately 200 µl of plant extracts were combined with 0.4 ml distilled water and 0.4 ml diluted Folin-Ciocalteu reagent. The mixture was then incubated in a boiling water bath for exactly 1 minute.

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Subsequently, the absorbance was measured at a wavelength of 650 nm using a spectrophotometer. The activity of the sample was estimated using a calibration curve of standard catechol. The results were expressed as mg of catechol equivalents per 100g of sample. [9]

➢ *Determination of Total Flavonoid Content:*

The total flavonoid content was determined using the AlCl3 colorimetric method. About 0.5 ml of sample solution was mixed with 1.5 ml of 95% methanol, 0.1 ml of 10% AlCl3, 0.1 ml of 1M potassium acetate (CH3COOK), and 2.5 ml of deionized water. After approximately 40 minutes at room temperature, the optical density (O.D.) of the reaction mixture was measured at 415 nm using a spectrophotometer against a deionized water blank. Quercetin was utilized as a standard, and the levels of total flavonoid contents in the different samples were determined in duplicate using a standard curve ranging from 0 to 50 mg. [10]

➢ *Antioxidant Assays:*

- *DPPH Free Radical Scavenging Assay:*

The stable radical DPPH was employed to assess the scavenging activity of the extracts, utilizing the DPPH test, which is well-suited for evaluating the antioxidant potential of crude extracts. Free radical scavenging activity was evaluated in vitro through a 1,2-diphenyl-2-picryl-hydrazyl (DPPH) assay. Specifically, 1.0 ml of 0.1 mM DPPH in methanol was mixed with varying concentrations of plant extract in equal volumes. After thorough mixing, the reaction mixture was incubated in darkness for 30 minutes. Absorbance was measured at 517 nm against a methanol blank. Ascorbic acid served as the standard for comparison. The higher scavenging activity was observed at lower wavelengths of the reaction mixture. The experiment was replicated twice to ensure consistency. IC50 values indicate the concentration of the sample needed to scavenge 50% of DPPH free radicals. the equations calculated the proportion of free radical inhibition or scavenging. %Inhibition = [(Absorbance of Control- absorbance of sample) /Absorbance of control] x 10 [11]

- *Assay of Nitric Oxide-Scavenging Activity:*

Kumaran and Karunakaran (2006) proposed a method for assessing nitric oxide-scavenging activity with slight modifications. In this method, 0.5 mL of samples were combined with 2.2 mL of phosphate-buffered saline at pH 7.4 (containing 545 mg Na2HPO4, 160 mg NaH2PO4, 4.5 g NaCl in 500 mL distilled water) and 0.1 mL of sodium nitroferricyanide (III) dehydrate in phosphate-buffered saline. After 150 minutes of incubation at room temperature, 0.2 mL of Griess reagent was added. The absorbance was measured at 546 nm after 20 minutes. Distilled water and curcumin were used as the control and standard, respectively, for the assay. The percent inhibitory activity was calculated using the formula [(A0 - A1)/A0] x 100, where A0 represented the absorbance of the negative control and A1 was the absorbance of the extract/curcumin. Linear regression analysis was employed to determine the sample concentration required to scavenge 50% of NO radicals (IC50).

➢ *Anti-glycation Assay:*

BSA was glycated using the described method with some modifications. BSA (1 mL, 10 mg/mL) was incubated with dilute extracts (1 mL) and fructose in potassium phosphate buffer saline (PBS) (2 mL, 200 mM, pH 7.4) containing 0.02% sodium azide, in the dark at 37°C for 4 days in sealed tubes under sterile conditions (filtered with a 0.22 μm filter). This mixture was referred to as the 'glycated sample' afterward. Negative control (1 mL BSA + 3 mL PBS) and positive control (1 mL BSA + 1 mL fructose + 2 mL PBS) were maintained under similar conditions. After incubation, unbound fructose was eliminated by dialysis against PBS, and the dialysate was used for further analysis. All additions and analyses were performed in triplicates. [12]

- *Fructosamine Assay:*

The concentration of fructosamine, the amadori product in glycated albumin samples and controls, was determined using the nitroblue tetrazolium (NBT) assay. A solution of NBT (0.75 mM) was prepared in carbonate buffer (0.1 M, pH 10.35). Subsequently, glycated samples (40 μL) were incubated with 0.8 mL of NBT solution at 37°C for 30 min. The absorbance was measured at 530 nm using a Genesys 10S UV-Visible spectrophotometer (Thermo Scientific). Fructosamine concentration was calculated using the standard 1-deoxy-1-moepholinofructose curve (Y ¼ 0.00X þ 0.017, r ¼ .981) and expressed in μM/mg of protein. [13]

- *Congo red Assay:*

As stated earlier, the method was applied to determine the coagulation amount in the glycated sample. Congo red solution (100 μM) was prepared in PBS (pH 7.4) containing ethanol (10%, v/v). Glycosylated samples (500 μL) were treated with the Congo red solution (100 μL) for 20 minutes before measuring the absorbance at 530 nm. [14]

➢ *Antidiabetic Assays:*

- *α-Amylase Inhibition Assay:*

An improved pancreatic-amylase inhibition assay, based on an earlier study, was employed. The mixture was incubated at 37°C for 10 minutes with various concentrations of test extracts (125 μL) and 125 μL of α-amylase mixture (0.5 mg/mL in 0.1 M sodium phosphate buffer, pH 6.9). The reaction was then further incubated for 30 minutes at 37°C. After incubation, 250 μL of dinitro salicylic reagent (1% 3,5-dinitro salicylic acid, 0.2% phenol, 0.05% Na2SO3, and 1% NaOH in aqueous solution) was added to stop the reaction. The reaction mixtures were heated at 100°C for 10 minutes, then mixed with 250 μL of potassium sodium tartrate solution (40%) to stabilize the color. The absorbance of the reaction mixture was measured at 540 nm after cooling to room temperature. Metformin (at different concentrations) was used as a positive control. All samples were analyzed twice. The percentage inhibition was calculated using the equation: Abs540(Control)-Abs540(Sample) /Abs540(Control)*100 = %Inhibition Abs540 (sample) represents the absorbance of maltose generated from starch by enzymes at 540 nm in the presence of the extracts, and Abs540 (Control) is the absorbance of the generated maltose at 540 nm when the extract is not present. The IC50 values

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were determined to assess the α-amylase inhibitory activities of various concentrations of the most effective extracts.[15]

- *α-Glucosidase Inhibition Assay:*

The α-glucosidase inhibition assay was adapted from a previous study. Briefly, sample extracts (at different concentrations, 50 µL) were mixed with yeast α-glucosidase enzyme (0.2 U/mL in 0.1 M potassium phosphate buffer solution, pH 6.9, 100 µL) and incubated at 37 °C for 30 min. After pre-incubation, the reaction mixture and p-nitrophenyl-α-D-glucopyranoside solution (5 mM, 50 µL) were further incubated at 37 °C for 30 min. Finally, sodium carbonate solution (0.1 M, 60 µL) was added to the reaction mixture and incubated again at 37 °C for 20 min. Readings of absorbance were taken at both the beginning and end of incubation at 405 nm. Metformin (0.25– 100 µg/mL) was used as a positive control. The percentage inhibition was calculated using the formula: %Inhibition = Abs540(Control)-Abs540(Sample) /Abs540(Control)* 100Abs405 (test) represents the absorbance of the generated p-nitrophenol by the enzymes at 405 nm in the presence of the test sample, and Abs405 (extract) is the absorbance of the extracted p-nitrophenol by the enzyme. Absorbance 405 (control) is the absorption of produced p-nitrophenol by the enzyme at 405 nm in the absence of the extract. The α-glucosidase inhibitory activities of various concentrations of the most effective extracts were assessed to determine IC50. [16]

➢ *Fourier Transform Infrared Spectrophotometer (FTIR):*

Based on the annotated spectrum, the absorbed light wavelength corresponds to characteristic chemical bonds. Chemical bonding within a molecule can be elucidated by analyzing the infrared absorption spectra. Fractions collected via column chromatography, exhibiting the highest α-amylase inhibitory potentials, were selected for FTIR analysis. For this analysis, 50 µl of the extract was directly applied to translucent sample discs and loaded into an FTIR spectroscope. The spectrum was scanned over a range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. [17]

➢ *UV-Visible Spectra Analysis :*

The absorption of ultraviolet or visible light by chemical substances produces distinct spectra, forming the basis for spectroscopy. At its core, spectroscopy explores the interactions between light and matter. UV-visible spectra were recorded in the range of 200 nm to 800 nm with a 1 mm path length quartz cuvette using a UV-Vis spectrophotometer.[18]

III. RESULTS

➢ *Collection of Sample: -*

Fig 1 *Costus Igneus* (Insulin)


Fig 2 Withania Coagulans (Pannerpushpam)


Fig 3 HolarrhenaAntidysenterica (Indrajav)


Fig 4 Fresh Cow Urine

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Plant samples were collected and then kept for shade-drying After that, it was ground using a mortar and pastel to form a fine powder. Also, fresh cow urine is collected and stored in dark airtight bottles.

➢ *Sample Preparation: -*

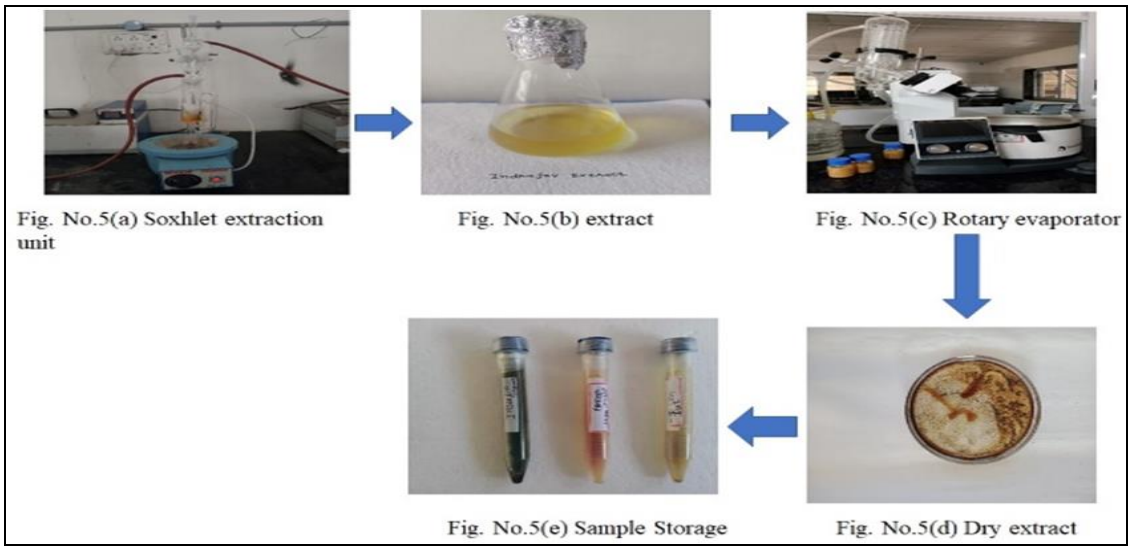

Fig 5 Sample Preparation

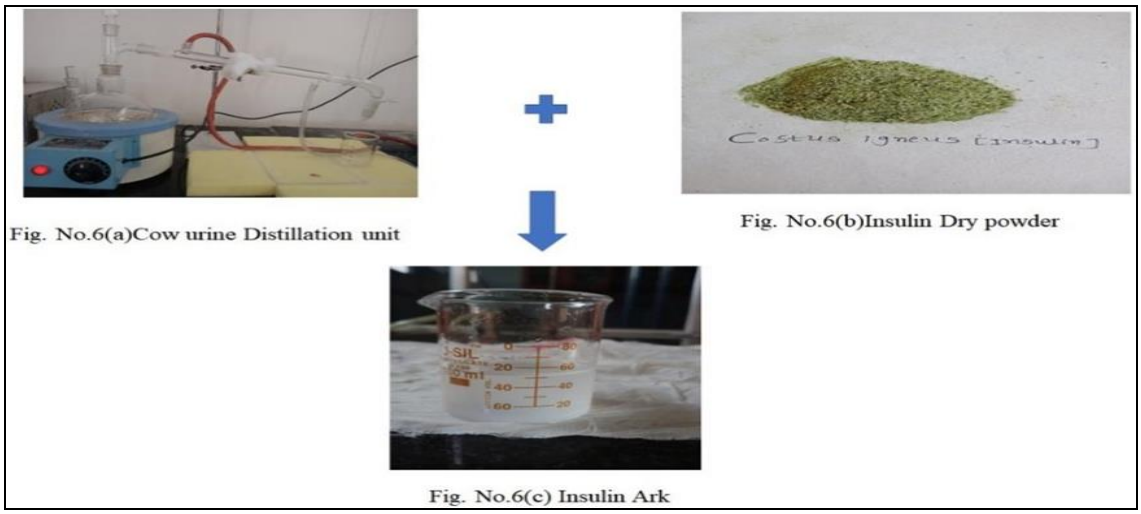

Fig 6 Sample Preparation

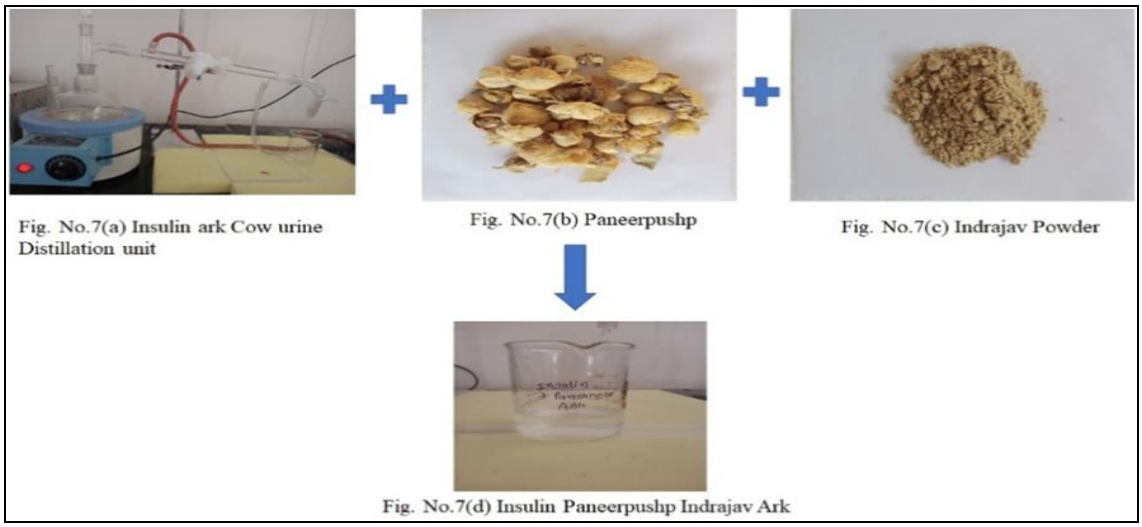

Fig 7 Sample Preparation

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As shown in the figure the collected samples were then powdered and stored as an Ark and extracted by using the Distillation And Soxhlet extraction technique.

➢ *Plant Extract and Ark Yield (%):*

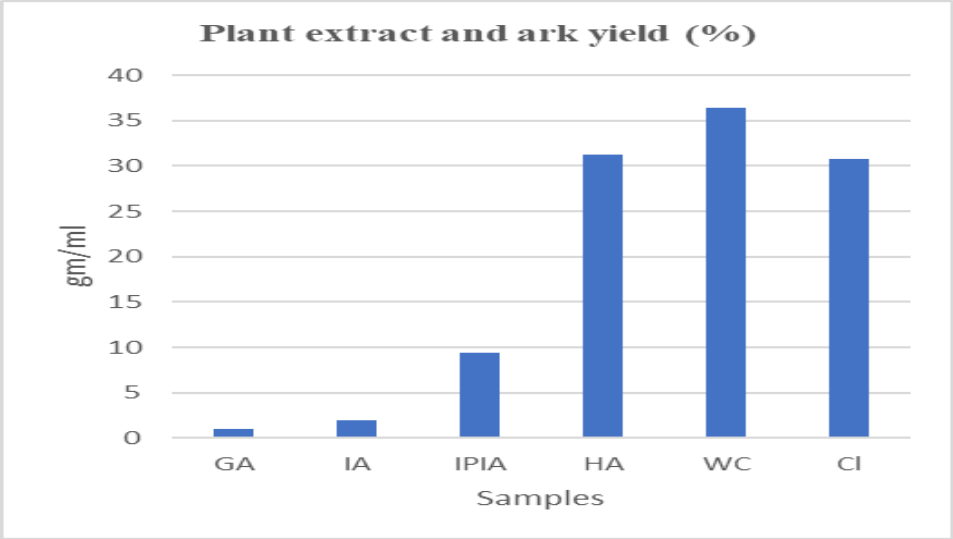

Fig 8 Plant extract and ark yield (%) of various samples

The crude Methanol extracts of these plant samples and cow urine distillation Ark were used for the analysis. The percentage yield of all samples is presented in Figure No. 1. The values ranged from 1% to 36.4% (w/v). Among these, WC exhibited the highest percentage yield, while GA yielded the lowest percentage.

Table 2 Sample Name with Code:

Sample code	Phenotypic Name	Common Name
GA	NA	Ark
IA	NA	Insulin Ark
IPIA	NA	Insulinpaneerpushamas Ark
HA	*Holarrhena Antidysenterica*	Indrajav
WC	*Withania coagulants*	Pannerpushpam
CI	*Costus Igneus*	Insulin

➢ *Qualitative Phytochemical Analysis:*

Table 3 Qualitative Phytochemical Analysis of Various Sample

Phytochemicals	Test	GA	IA	IPIA	HA	WC	CI
Tannin	Fecl3 test	+	+	-	-	-	-
Alkaloids	Wegner's test	+	+	-	+	+	+
Carbohydrate	Molish test	+	+	-	+	+	+
	Benedict's test	+	+	-	+	+	-
Protein	Biuret test	+	+	-	-	-	-
	Ninhydrin Test	+	+	-	-	+	-
Phenols	FCR test	+	+	-	+	+	+
Anthocyanin	NaOH test	-	-	-	-	+	+
Flavonoids	AlCL3 test	+	+	-	+	+	+
Saponins	Foam test	-	-	-	-	+	-
Sterol	L.B. test	-	-	-	-	-	+

Qualitative phytochemical analysis of extraction urine distillation arks containing various constitutes are represented in Table The phytochemical analysis of the Methanolic extracted HA, WC, CI, and cow urine distillation arks like GA, IA, and IPIA showed the presence of biologically active secondary metabolites compound. The qualitative phytochemical analysis of the methanolic extracts from the samples revealed the presence of primary and secondary metabolites such as alkaloids, steroids, and cardiac glycosides. These detected preliminary phytochemicals are known to possess medicinal and health-promoting properties.

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Similarly, the qualitative phytochemical analysis of the methanolic extracts and cow urine distillation arks from the samples showed the presence of primary and secondary metabolites, including alkaloids, steroids, and cardiac glycosides. These preliminary phytochemicals are also known to have medicinal and health-promoting properties.

Various mechanisms of action have been proposed for these plant extracts, including their effects on the activity of pancreatic ß cells (synthesis, release), increased insulin sensitivity, or insulin-like action. The most commonly involved active constituents are flavonoids, tannins, phenolics, and alkaloids.

Table 3 Physicochemical Analysis of Cow Urine:

Test	Result
Temperature	38.5℃
pH	8.63
Color	Light yellow
Odor	Pungent astringent
TDS	0.01gm/1ml

- *Determination of Total Phenol Content:*

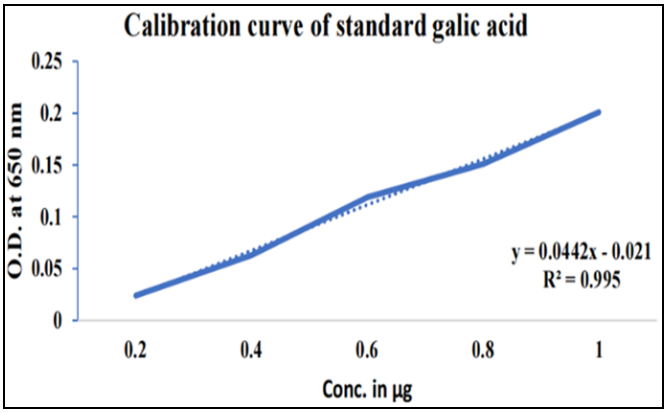

Fig 9 std Gallic Acid for TPC

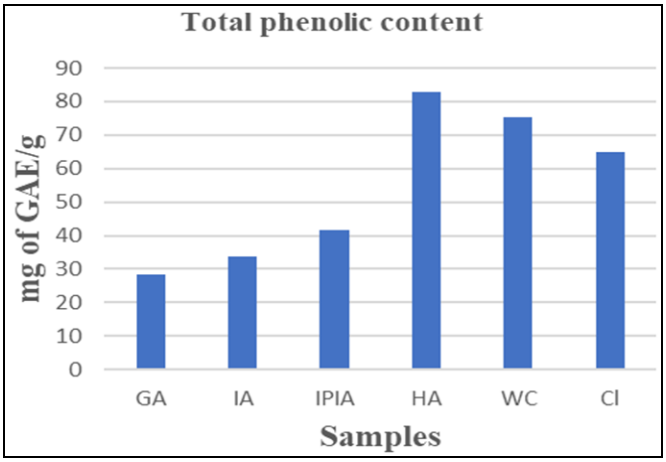

Fig 10 Total Phenolic Content of Various Sample

The total phenolic content of the methanolic extracts from samples HA, WC, CI, and cow urine distillation arks such as GA, IA, and IPIA was estimated using the standard Gallic acid equivalent of phenols. phenolic compounds in plants contain the hydroxyl group, which acts as a good scavenger and is known as a powerful chain-breaking antioxidant (Vanitha *et al*., 2016). Indeed, plant phenols serve as essential nutritional antioxidants, possessing an optimal structural chemistry that allows them to efficiently scavenge free radicals. (Kamath *et al*., 2015). Our data represents phenolic content of HA and WC have higher phenolic content whereas GA and IA have comparatively lower phenolic content.

- *Determination of Total Flavonoids content:*

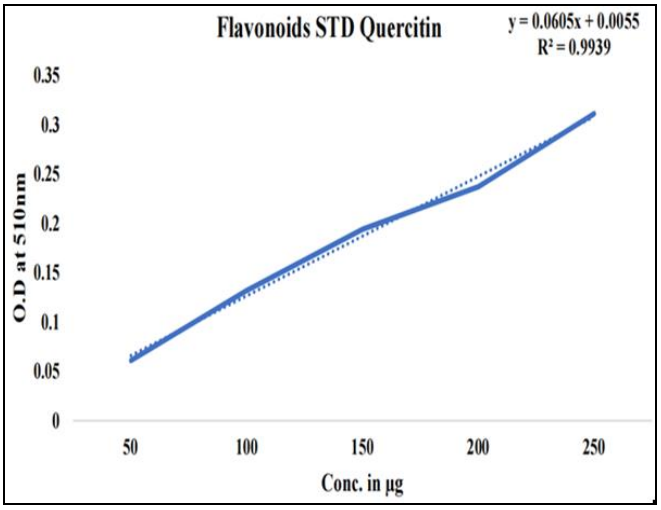

Fig 11 Standard graph of Quercetin

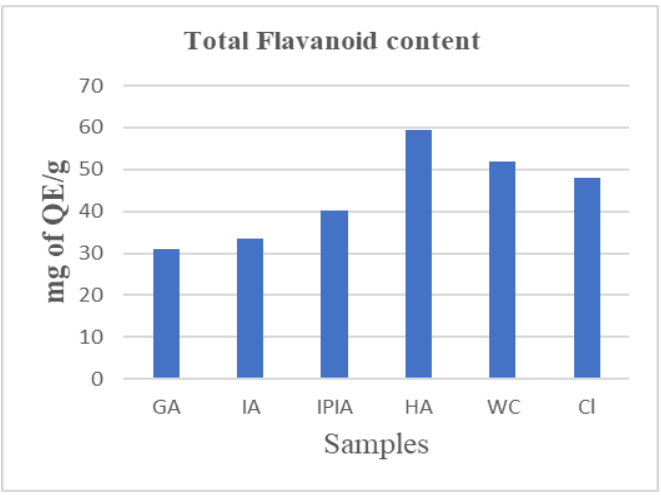

Fig 12 Total Flavonoids Content Various Sample

The total flavonoid content of Methanol extracts of HA, WC, CI, and cow urine distillation arks like GA, IA, and IPIA was estimated using the standard quercetin equivalent of flavonoid. The quercetin calibration curve was plotted with different concentrations. Flavonoids, potent antioxidants, exhibit characteristics such as scavenging free radicals, chelating metals, and inhibiting lipid peroxidation, depending on the number and configuration of phenolic hydroxyl groups in the molecules and on the glycosylation and configuration of other substituents. (Baba *et al*., 2014). Our data represents the flavonoid content of HA and WC as higher and GA and IA have lower total flavonoid content.

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➢ *Antioxidant Assays:*

- *DPPH Free Radical Scavenging Assay:*

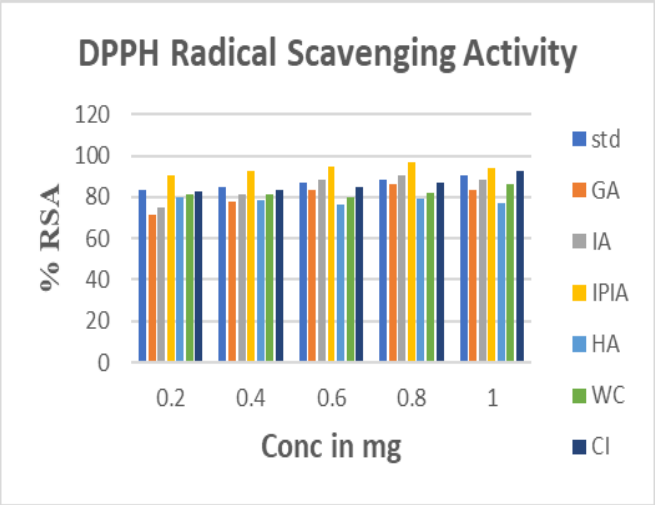

Fig 13 DPPH Radical Scavenging Activity of Various Sample

Our data indicate that the percentage of scavenging effect of DPPH radical increased in parallel with the concentration of both the extracts and arks. From the results, it is evident that the species possess hydrogen-donating capabilities for all extracts and arks, exhibiting free radical scavenging activity. The highest percentage of radical scavenging activity was found in IPIA and CI, as presented by evaluating antioxidant efficiencies, known as IC50. A lower IC50 number indicates a greater overall effectiveness of the antioxidant samples.

The degree of discoloration shows the extract's scavenging capability. The antioxidant effect on the scavenging of DPPH radicals was attributed to the capacity of the antioxidants to donate hydrogen. DPPH neutralizes its free radical character through the transfer of an electron or a hydrogen atom to antioxidants. (Garg *et al*., 2012).

As a result, it is the most straightforward and dependable approach for detecting radical scavenging activity.

Also, cow urine-mediated ark has volatile fatty acids that function as antioxidants Compounds such as acetic acid 2-propenyl ester, acetic acid methyl ester, 2,2,3-trichloro propionic acid, and butanoic acid act as antioxidants. [1]

- *NO Scavenging Activity:*

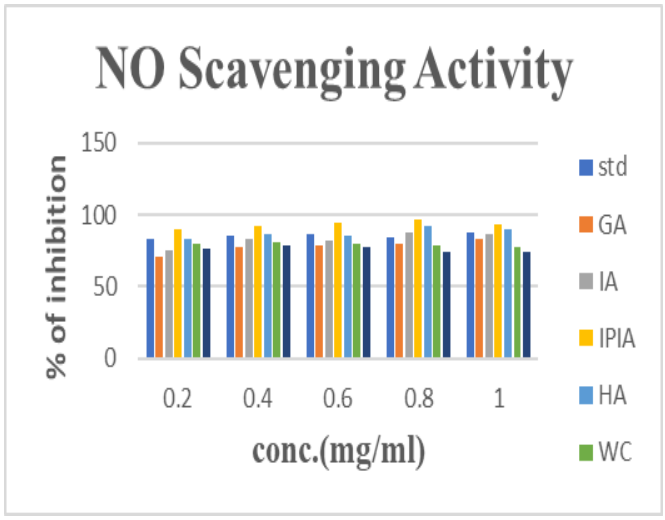

Fig 14 NO Scavenging Activity Various Sample

Our data indicate that the percentage of scavenging effect of DPPH radical increased in parallel with the concentration of both the extracts and ark. From the results, it is evident that the highest percentage of radical scavenging activity was found in IPIA and HA.

The extract and ark may possess the ability to counteract the effects of NO formation, which could be beneficial in preventing the negative consequences of excessive NO production in the human body. Furthermore, the scavenging activity may help halt the chain of reactions triggered by excess NO production that are harmful to human health. Additionally, because the extract lowered the concentration of catalytic transition metals in lipid peroxidation, it exhibited considerable metal-chelating ability[19]. Chelating compounds are valuable as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized state of the metal ion, according to research.[20].

There is a documented correlation between the levels of phenolic compounds and the antioxidant capacity of plants. Phenolic compounds exert their protective function through various mechanisms, such as acting as blocking agents to inhibit the formation of carcinogens from precursors. Additionally, compounds abundant in flavonoids have been observed to possess the intrinsic ability to modulate the body's responses to allergens, viruses, and carcinogens.

Also, cow urine-mediated ark has volatile fatty acids that function as antioxidants, Some examples of these compounds include 3-methyls, propyl ester, 1H indol-3-acetate, and acetic acid phenyl ester, which function as antioxidants. [1]

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➢ *Antiglycation Activity:*

- *Fructose Amine Inhibition Assay*

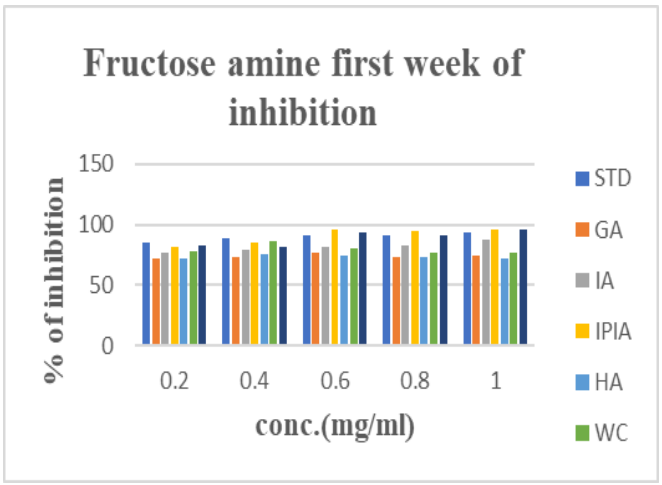

Fig 15 Fructose Amine Inhibition First Week

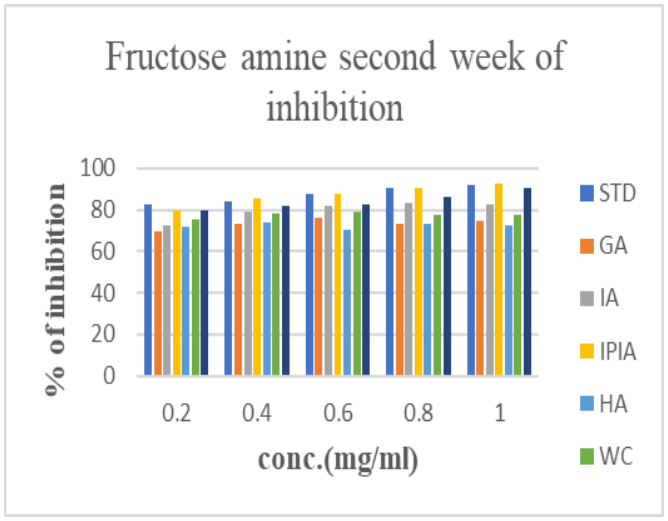

Fig 16 Fructose Amine Inhibition Second Week

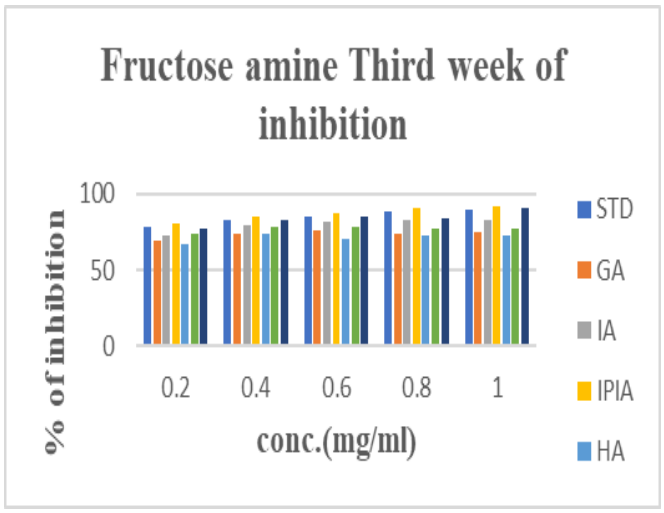

Fig 17 Fructose Amine Inhibition Third Week

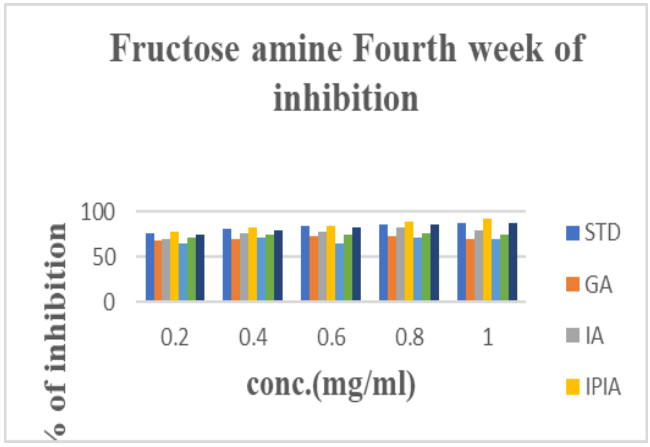

Fig 18 Fructose Amine Inhibition Fourth Week

Figure 15,16,17,18 shows the effects of Aminoguanidine and HA, WC, CI, GA, IA, and IPIA on the deter of AGE formation. At 7, 14, 21, and 28 days, the deterrence of AGE formation in fructose-glycated BSA was noted, indicating a significant increase in the percentage inhibition of AGEs formation. Each value represents the mean. The results demonstrated that Aminoguanidine significantly decreased the formation of AGEs in a concentration-dependent manner (Ahmad et al., 2013b). Remarkable inhibition of AGEs formation was observed, with the highest inhibition seen in Aminoguanidine at concentrations of 0.2, 0.4, 0.6, 0.8, and 1 compared to the control at 7 and 14 days. By day 28, there was a noticeable trend of increased inhibitory activity. Also, cow urine-mediated ark has volatile fatty acids that function as antioxidants[1].

- *Congo Red Binding Inhibition Assay:*

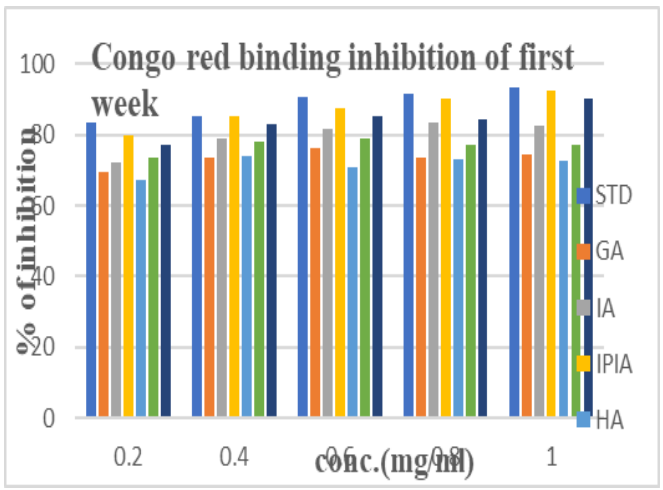

Fig 19 Congo Red Binding Inhibition of the First Week

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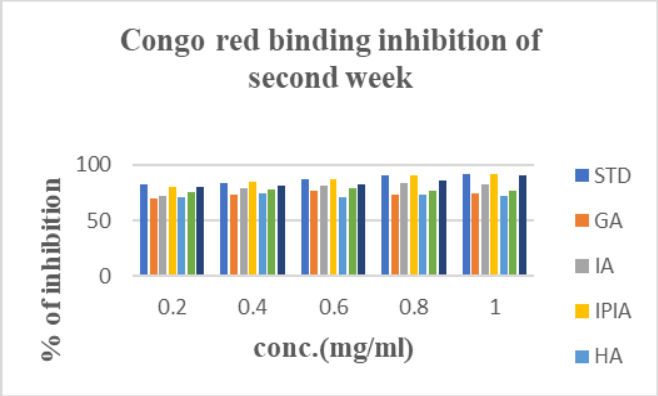

Fig 20 Congo Red Binding Inhibition of the Second Week

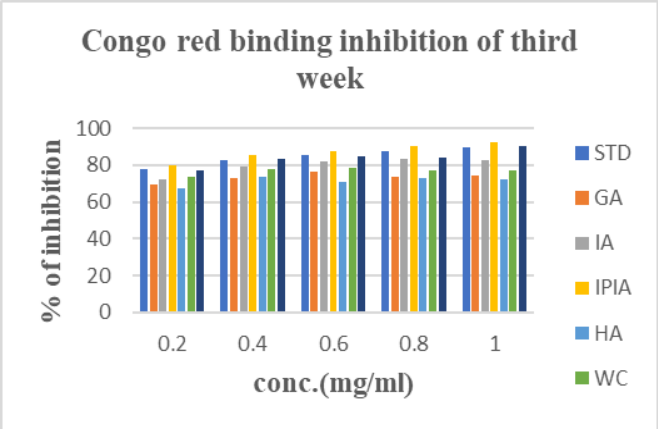

Fig 21Congo Red Binding Inhibition of the Third Week

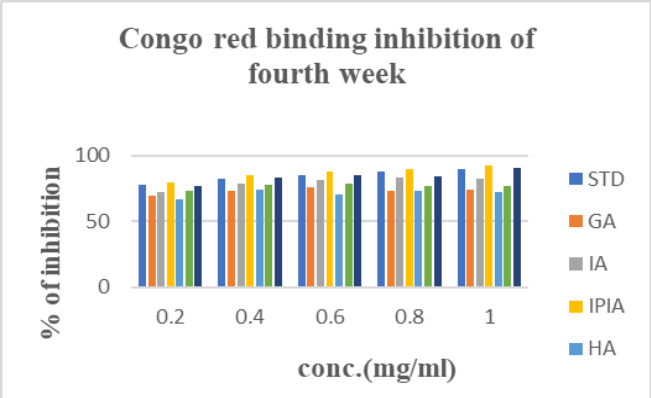

Fig 22 Congo Red Binding Inhibition of the Fourth Week

Figure 19,20,21,22 shows the effects of Aminoguanidine and HA, WC, CI, GA, IA, and IPIA The inhibition of AGEs formation in fructose-glycated BSA was observed at 7, 14, 21, and 28 days, indicating a significant increase in the percentage inhibition of AGEs formation. Each value represents the mean. The results demonstrated that Aminoguanidine significantly reduced the formation of AGEs in a concentration-dependent manner. Remarkable inhibition of the formation of AGEs was observed, with the highest inhibition seen in Aminoguanidine at concentrations of 0.2, 0.4, 0.6, 0.8, and 1 compared to the control at 7 and 14 days. By day 28, there was a noticeable trend of increased inhibitory activity. (Armstrong *et al*., 2010). Also, cow urine-mediated ark has volatile fatty acids that function as antioxidants, [1].

➢ *Antidiabetic Activity:*

- *A Amylase Inhibition*

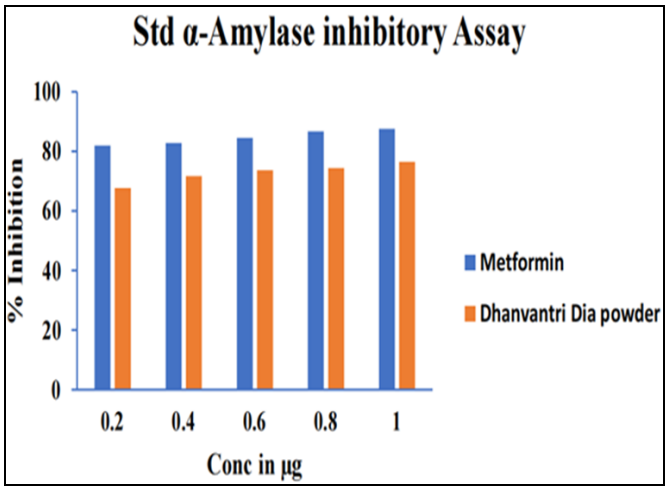

Fig 23std α Amylase Inhibition

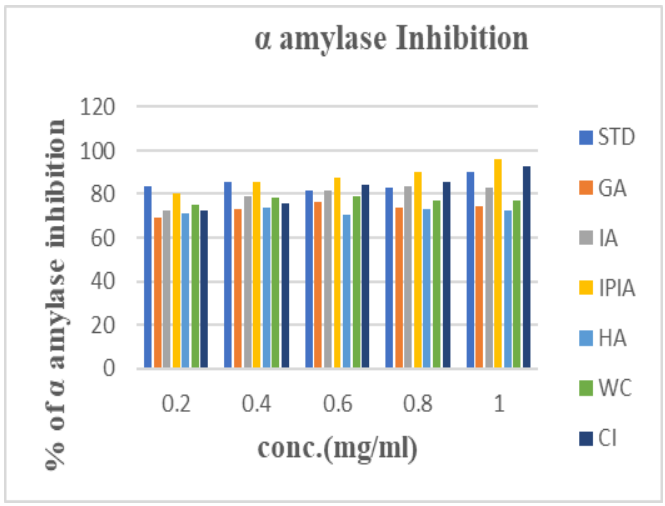

Fig 24 α Amylase Inhibition of Various Sample

Indeed, alpha-amylase plays a crucial role in the digestive process due to its ability to break down polysaccharides, such as starch, into smaller sugar molecules. This enzyme is produced primarily in the pancreas and salivary glands and acts on complex carbohydrates, initiating their digestion into simpler sugars like maltose and glucose, which can then be absorbed by the body. It is present largely in saliva and digestive juices. One prospective option for preventing excessive postprandial blood glucose levels is to target and inhibit this enzyme. (Ademiluyi & Oboh, 2013). The highest % of inhibition is shown in IPIA which is demonstrated in the graph. Graph 14 illustrates the alpha-amylase inhibition potential of the samples. It suggests a dose-dependent relationship, indicating that as the concentration of the extract increases, the inhibition of the enzyme also increases. This observation highlights the ability of the samples to effectively inhibit the activity of alpha-amylase, which is crucial for controlling blood sugar levels by reducing the breakdown of complex carbohydrates into glucose.

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- *In Vitro A- Glucosidase Inhibition Assay:*

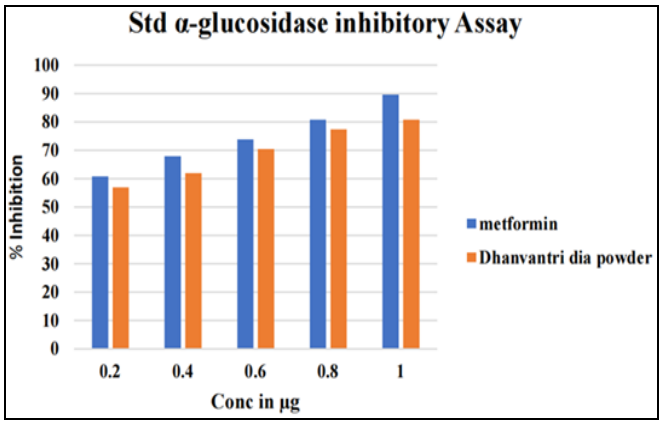

Fig 25 α-Glucosidase std Inhibition Assay

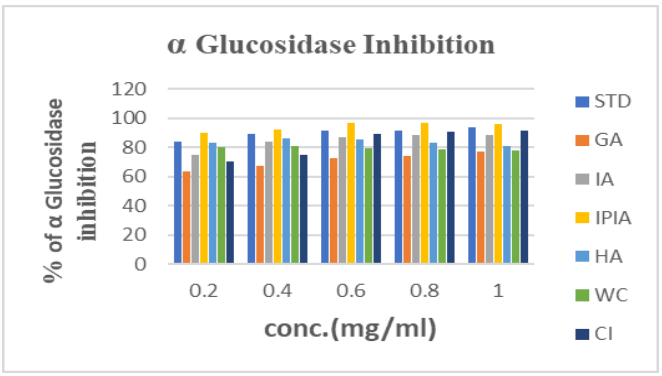

Fig 26 α-Glucosidase Inhibition Assay of Various Sample

The α-glucosidase enzyme, located on the mucosal brush border of the small intestine, plays a crucial role in carbohydrate digestion. Inhibiting this enzyme can effectively delay glucose absorption, thereby preventing spikes in postprandial blood glucose levels. This inhibition may offer a potential strategy for managing diabetes and slowing its progression. and its inhibition. Its function is to convert and degrade complicated carbs into tiny, simple, and digestible ones. [21]. The highest % of inhibition is shown in IPIA and CI which is demonstrated in Figure No. 26 Cow urine-mediated ark has volatile fatty acids that function as antioxidants, These compounds, including acetic acid 2-propenyl ester, acetic acid methyl ester, 2,2,3-trichloro propionic acid, butanoic acid, 3-methyls, propyl ester, 1H-indol-3-acetate, and acetic acid phenyl ester, are known to possess antioxidant properties. among others [1].

➢ *Fourier Transform Infrared Spectrophotometer (Ftir):*

Indeed, Fourier Transform Infrared Spectroscopy (FTIR) is widely regarded as one of the most effective techniques for identifying the types of covalent bonds (functional groups) present in compounds.

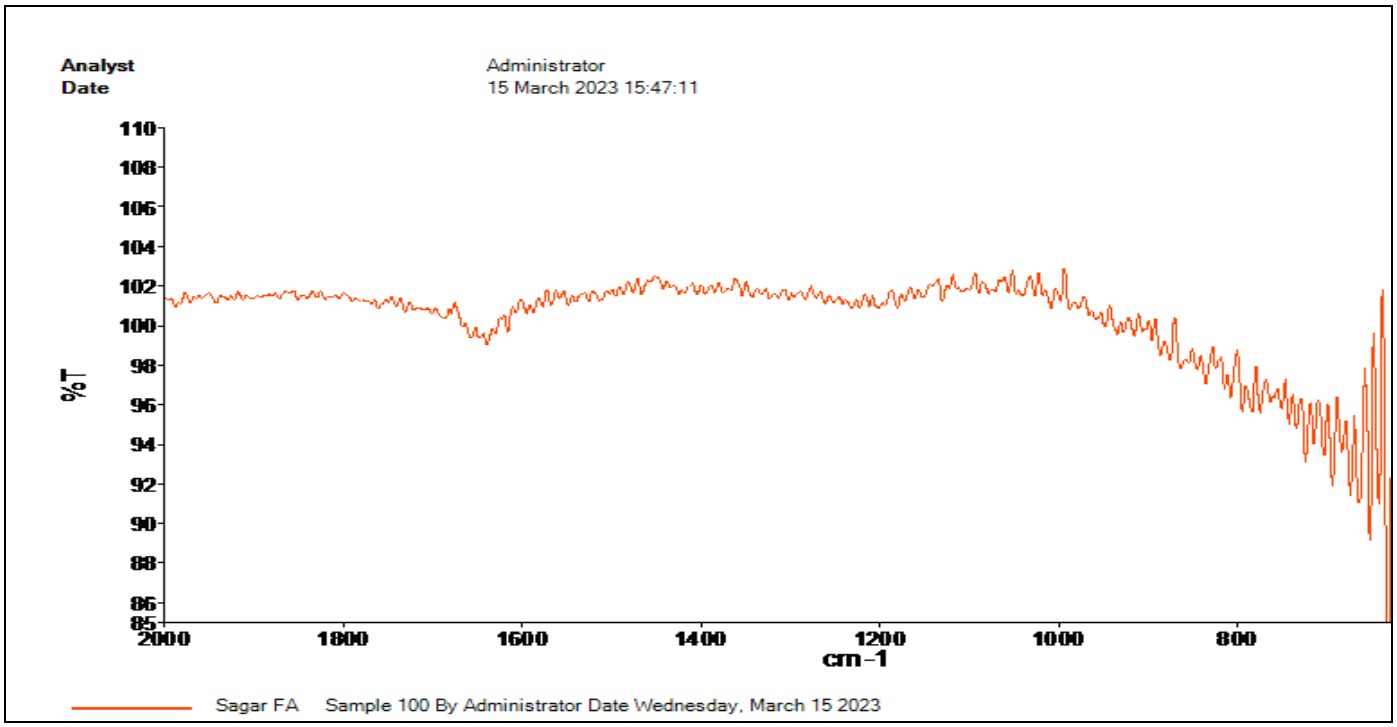

Fig 27 FTIR Spectrum of IPIA

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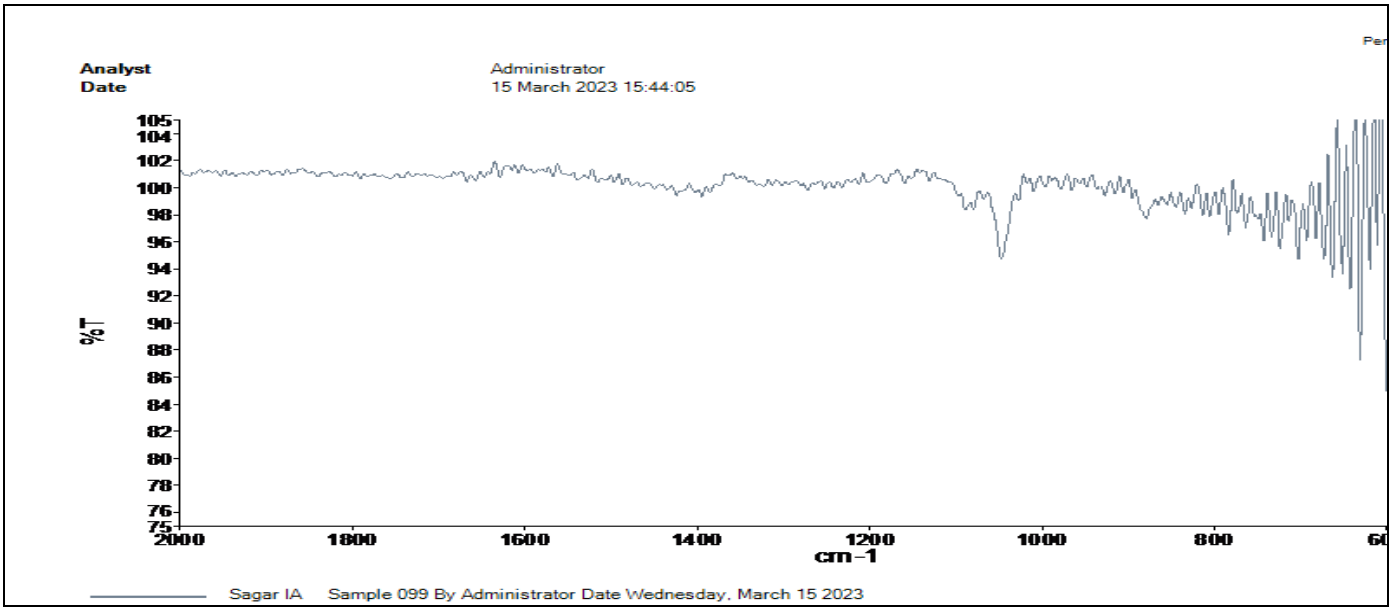

Fig 28 FTIR Spectrum of IA

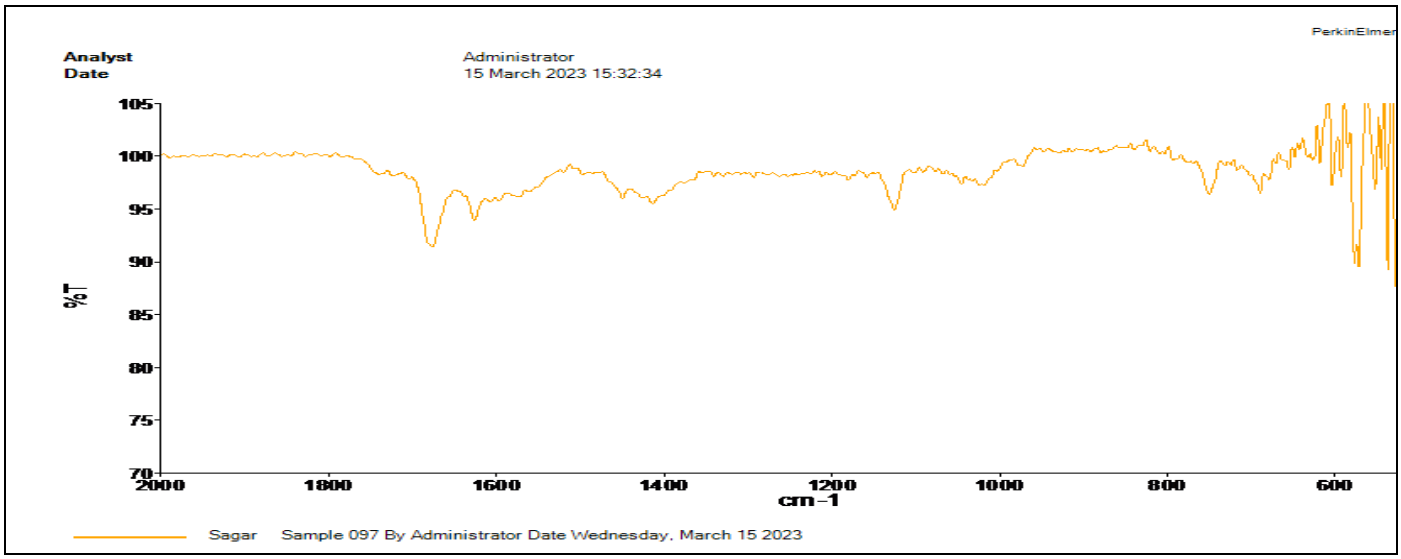

Fig 29 FTIR Spectrum of GA

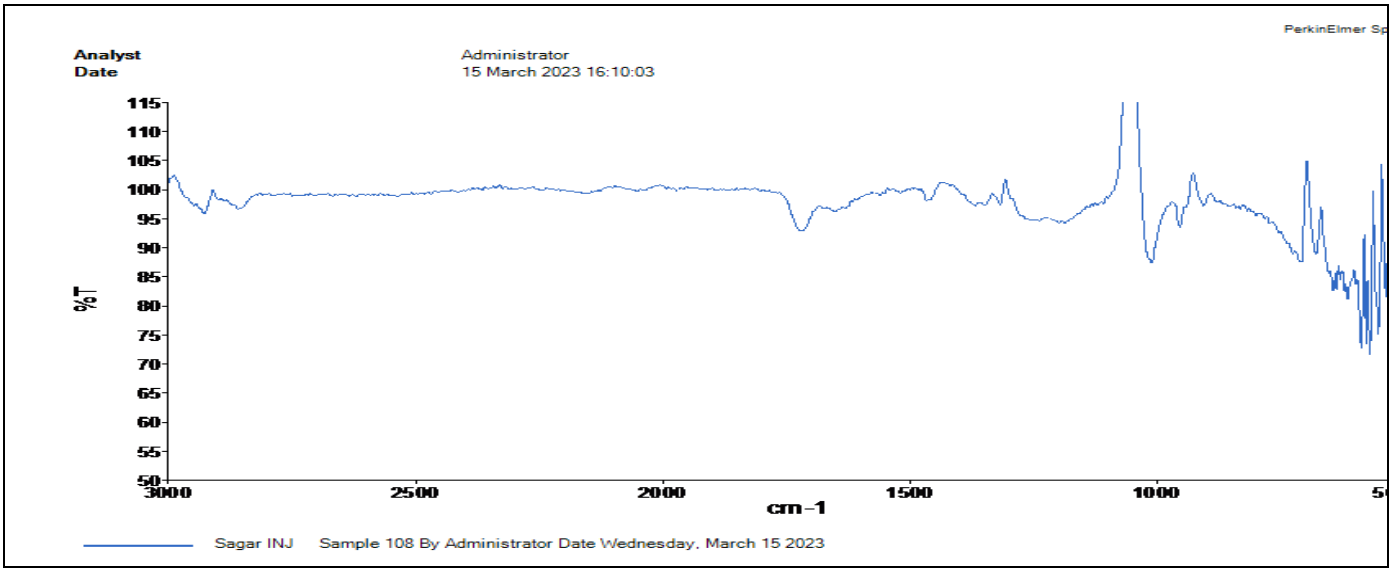

Fig 30 FTIR Spectrum of INJ

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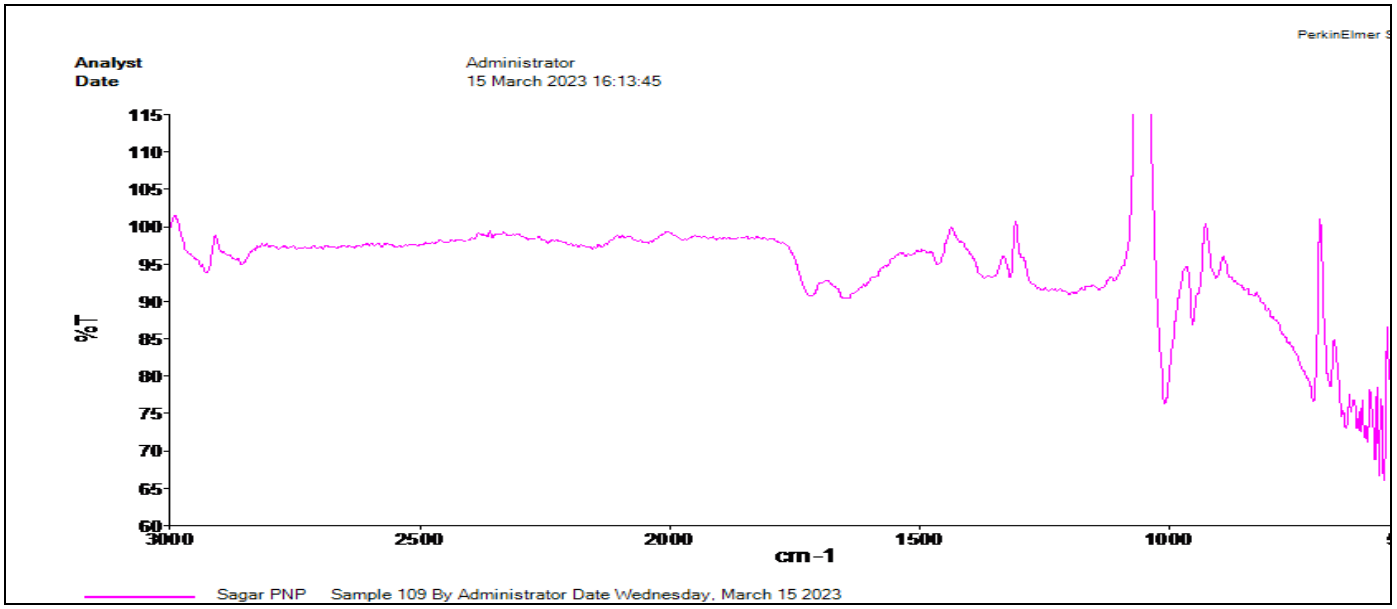

Fig 31FTIR Spectrum of PNP

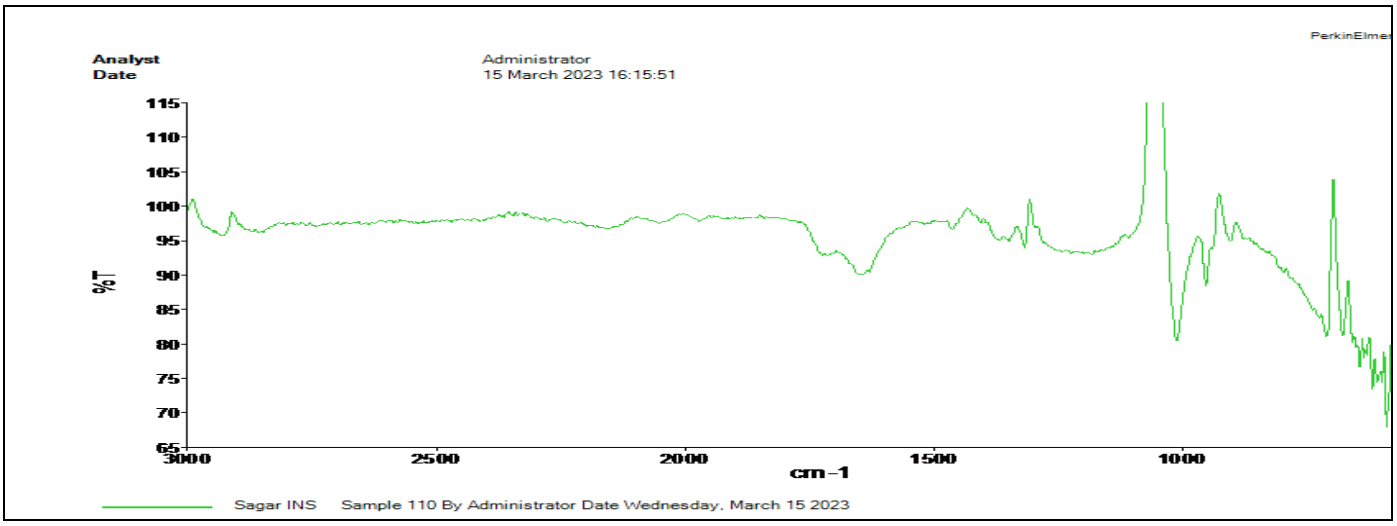

Fig 32 FTIR Spectrum of INS

The x-axis, or horizontal axis, of the infrared spectrum, represents the intensity of the infrared spectra. It illustrates how the sample responds when exposed to the infrared portion of the spectrum. the peaks, also known as the electromagnetic spectrum correspond to absorbance bands, representing the different vibrations of the sample's atoms. In mid-range IR spectroscopy, the wavenumber on the infrared spectrum typically ranges from 4,000 to 400 cm^-1. The y-axis, or vertical axis, represents the amount of the substance under examination that absorbs or transmits infrared light.

The functional groups frequently detected in FTIR (Fourier-transform infrared spectroscopy) in the following wavenumber areas are:

This is the fingerprint area, and it comprises several tiny peaks that may be utilized to identify particular functional groupings.

Table 4 Stretching and Functional Group Analysis of Various Samples at Transmission Band

Transmission band	Stretching
580-750 cm^-1	show a halogen (e.g., chlorine or bromine) in the structure.
1000 cm-1	C-O (carbon-oxygen) stretching in ethers, alcohols, and phenols
1060 cm-1	C-N (carbon-nitrogen) stretching in primary amines
1640 cm-1	C=C (carbon-carbon double bond) stretching in alkenes
1700 cm-1	C=O (carbonyl) stretching in ketones, aldehydes, carboxylic acids, esters, and amides
1750 cm-1	C=O (carbonyl) stretching in conjugated ketones, anhydrides, and acid chlorides
2800-2900 cm-1	C-H (carbon-hydrogen) stretching in alkanes, alkenes, and alkynes

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It is important to remember that the exact placement and intensity of the peak will most likely be determined by the building and its structural context. Different groups may also have absorption peaks in comparable places, hence it is crucial to examine the entire FTIR spectrum of a molecule while analyzing the findings.

➢ *UV-Visible Spectra Analysis:*

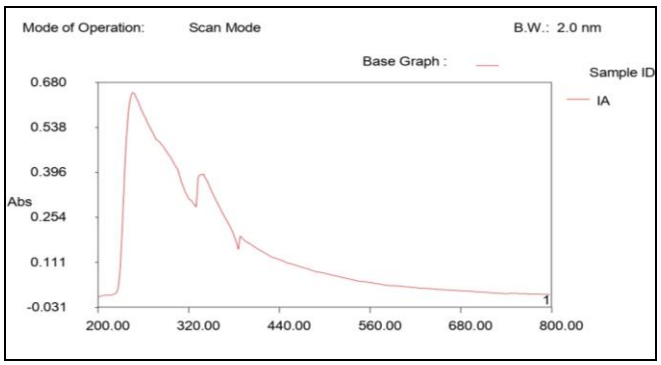

Fig 33 UV-Visible Spectra of GA

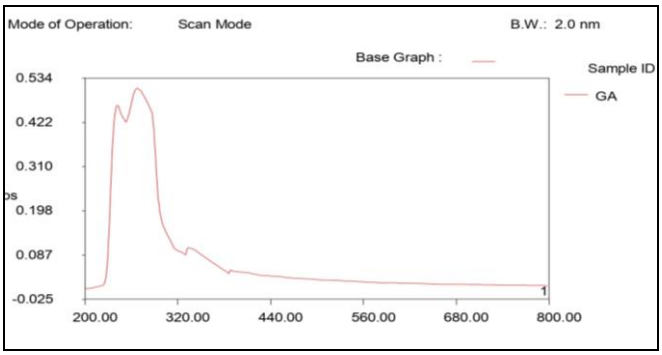

Fig 34 UV Visible Spectra of IA

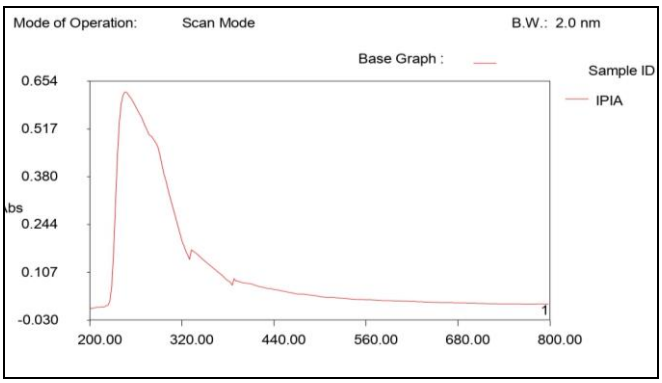

Fig 35 UV-Visible Spectra of IPIA

For the GA sample, the solution was subjected to UV-visible radiation using a UV-visible absorption spectrophotometer 117, with a resolution of ±1 nm, scanning between 200-800 nm at a speed of 200 nm/min. The absorbance of the solution was recorded, and maximum absorption was found at 230nm and 270nm. also for the IA sample maximum absorption was found at 340nm. also for the IPIA sample, the maximum absorption was found at 230. so by analyzing the graph, it represents properties and composition, as it can be used to identify the sample and determine its concentration.

IV. CONCLUSION

In conclusion, the present study aimed to evaluate the antioxidant, antiglycation, and anti-diabetic properties of cow urine ark formulated with medicinal plants such as insulin, panner-push, and indrajv. The individual and combined effects of these plants with cow urine were assessed, and the results indicated that the ark had higher antioxidant, antiglycation, and anti-diabetic properties than individual plants.

Moreover, this study highlights the importance of traditional knowledge in developing natural remedies for various ailments. The combination of medicinal plants with cow urine may enhance their therapeutic potential and provide a sustainable approach to health and wellness.

Overall, the findings of this study provide valuable insights into the potential of cow urine ark and medicinal plants in developing natural remedies for various diseases. Further research is warranted to explore the efficacy of these formulations in pre-clinical and clinical studies.

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