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ANTIOXIDANT AND ANTIGLYCATION POTENTIAL OF DEVELOPED POLYHERBAL FORMULATIONS AND EXTRACTED PHYTOCHEMICALS

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Abstract
Diabetes mellitus is a chronic metabolic disorder characterized by insulin deficiency or its ineffectiveness though produced by the body. More than 90% of patients affected by diabetes are of type II. Advanced Glycated End products (AGEs) in correlation with oxidative stress are mainly responsible for the long-term complications of diabetes. The present study was designed to evaluate antioxidant and antiglycation properties of three individual plants including *Trillidium goanum*, *Centorium tenuiflorum and Rubinia pseudoacacia* as well as polyherbal formulations (n=11). Results of crude extracts revealed highest antioxidant and antiglycation activity of polyherbal formulation S22 (*Centorium tenuiflorum+Morchella conica*) with IC_{50} values of 21.63±0.15µg/mL and 10.63±1.72µg/mL respectively. When the data was compared for phytochemicals, it was noticed that phenols and tannins from S28 (*Trillidium goanum+Centorium tenuiflorum+Morchella conica+Rubinia pseudoacacia*) showed the potent antioxidant activity by showing IC_{50} values of 7.6±0.36µg/mL and 8.17± 1.69 µg/mL respectively. However, for antiglycation properties phenols and tannins for S28 and S29 were found to be potent. The IC50 values were 7.046±3.19µg/mL (phenols, S28), 7.33±2.2µg/mL (tannins, S28), 5.34±1.86µg/mL (Phenols, S29) and 58.07±1.59µg/mL (tannins, S29). Additionally, quantification data indicated highest concentrations of phenols, flavonoids, and tannins in polyherbal formulation S28 which gives an indication of highest antioxidant and antiglycation potential of formulation S28.

Keywords: Diabetes Mellitus, antioxidant, AGEs, polyherbal formulations, antiglycation

1.0 Introduction
Diabetes mellitus is a metabolic disorder which arises from complex interactions between multiple genetic, environmental and lifestyle factors [1 & 2]. This chronic disease is characterized by hyperglycemia due to defective insulin secretion, insulin action or both. According to a systemic review in 2019 the prevalence of diabetes is 69% in developing countries and 20% in developed

countries which generates a significant socio-economic burden in developing countries [3]. Due to this high prevalence, the mortality ratio is high in the poor countries where people suffer due to low income. Mostly patients with diabetes mellitus have either type 1 diabetes (which is immune-mediated or idiopathic) or Type 2 DM. More than 90% of patients with diabetes are suffering from type 2 hence considered as the most prevalent type of diabetes. Although diabetes is not inherently harmful, it can cause several harmful side effects, such as microvascular and macrovascular disorders, which may eventually result in retinopathy, neuropathy, diabetic stroke, cardiovascular issues, dementia, depression, lower limb amputation and sexual dysfunction [4, 5 & 6].

Diabetic complications are greatly enhanced with increased NEG (non-enzymatic glycosylation) and oxidative stress [7& 8]. The free radical's formation in diabetes is due to glucose oxidation, non-enzymatic glycation, and oxidative degradation of amino acids [9]. Free radicals' generation in living systems can cause pathological conditions by damaging tissue and biomolecules in cellular level leading to degenerative diseases [10]. Synthetic antioxidants have adverse side effects compared to plants. The phytochemicals flavonoids, polyphenol, carboxylic acids, sterols and triterpenoid, tannins, carotenoids, alkaloids, and micronutrients are the best antioxidants [11 & 12]. There is an increasing demand for antioxidants from nature because phytochemicals are free radical scavengers. AGEs inhibitors could be potential therapeutic candidates in the prevention of macro- and micro complications in diabetes, as AGEs generate pro-inflammatory cytokines through enhanced activation of receptor for Advanced Glycated Endproducts (RAGE) [13]

Type 2 diabetes requires a combination of therapies and recent advances are being made for devising effective prophylactic and therapeutic protocol for managing the disease [14]. The costs of diabetes treatment and its related complications are staggering with conventional therapeutics being expensive, prone to side effects and rarely being available in most of the developing or underdeveloped countries [15]. Although many oral hypoglycemic drugs such as sulphonyl urea, thiazolidinediones, peptide analogs are available for the treatment of type 2 diabetes mellitus, but these have limitations [16]. Herbal drugs are proved to be a better choice over synthetic drugs because of less side effects.

According to world ethnobotanical data, 800 medicinal plants are used for the prevention of diabetes mellitus. Clinically proven that only 450 medicinal plants possess anti diabetic properties from which 109 medicinal plants have complete mode of action. There is a long history of traditional plants used for the control of diabetes in India and China. Herbal formulations are easily available without prescription. These herbal drugs are used for life threatening disease and have several advantages over synthetic drugs. These drugs are also used when chemical drugs are ineffective in treatment of disease. These are natural and thus nontoxic as compared to synthetic drugs. Herbal formulations contain natural herbs, fruits and vegetables extract which are beneficial in treatment of various diseases without any adverse effects. On the other hand, chemical drugs are prepared synthetically and have side effect also. Herbal formulations are ecofriendly and cheap as compared to allopathic medicines which are produced from chemicals and chemically modified natural products [17]. In the present work, antiglycation and antioxidant activities of three selected medicinal plants *Trillidium goanum*, *Centorium tenuiflorum* and *Rubinia pseudoacacia* and polyherbal formulations (n=11) were evaluated. In addition, phytochemicals including flavonoids, tannins and phenols were also extracted, quantified, and evaluated for their antioxidant and antiglycation properties.

2.0 Materials and methods

2.1 Plant collection

The medicinal plants were collected from the different areas of Khyber Pakhtunkhwa Pakistan in the month of August 2018. The selected plants were identified by Dr. Mohib Shah, Department of Botany, Abdul Wali Khan University Mardan, Pakistan. The specimen was deposited in herbarium of Department of Botany, Abdul Wali Khan University Mardan, Pakistan.

2.2 Sample preparation

The shed dried plants were ground into a fine powder and a total number of 11 polyherbal formulations were developed by mixing individual plants in specified ratios as shown in Table 1. Samples were soaked in methanol (1:10) for three successive days then centrifuged and filtered. The process was repeated thrice. Crude methanol extracts were stored at 4°C for further analysis.

Table 1: List of selected individual plants and developed polyherbal formulations

S/NO	Sample	Formulations	Ratios
1	S1	*Trillium govanianum*	S1
2	S2	*Centaurium tenuiflorum*	S2
3	S22	*Centaurium tenuiflorum+ Morchella conica*	1:1
4	S23	*Chichorium intybus + Trigonella foenum graecum + Saussarea lappa + Lipidium sativum + Nigella sativum*	2: 1:1:1:4
5	S24	*Nigella sativum+Morchella conica*	3:1
6	S25	*Nigella sativum +Morchella conica*	1:3
7	S26	*Nigella staivum+Morchella conica*	1:1
8	S27	*Trillium govanianum + Centaurium tenuiflorum + Morchella conica*	1:1:1
9	S28	*Trillium govanianum + Centaurium tenuiflorum + Morchella conica + Robinia pseudoacacia*	1:1:1:1
3	S29	*Trillium govanianum+ Robinia pseudoacacia*	1:1
11	S30	*Centaurium tenuiflorum + Robinia pseudoacacia*	1:1
12	S31	*Centaurium tenuiflorum + Robinia pseudoacacia*	1:3
13	S32	*Centaurium tenuiflorum + Robinia pseudoacacia*	3:1
14	NP	*Robinia pseudoacacia*	NP

2.3 Bioactive compound determination

2.3.1 Determination of Flavonoids

Flavonoid content was extracted by dissolving 20g of each sample in 200mL of 80% aqueous ethanol and placed in an incubator for 24 hours. Samples were than centrifuged and filtered using Whatman No.1 filter paper. Pellets were discarded and filtrate containing flavonoids were stored at 4ºC for further analysis. Flavonoids were determined using a standard protocol based on spectrophotometric assay as described earlier [18]. With slight modification. 250ul of flavonoid extract was mixed with 1.25mL of distilled water and 75ul of 5% NaNO2 solution. After 5 min, 150ul of a 10% AlCl3.H_2O was added and incubated for 6 min. The mixture was then treated with 500ul of 1M NaOH and 275ul of distilled water. The solution was well mixed using a vortex, and the absorbance at 415 nm was measured. Blank containing 80% aqueous ethanol was prepared by using the same method. For calculating the standard curve different concentrations of quercetin (15ug – 500ug) were used.

2.3.2 Determination of Total Phenol Content

Total phenol content was extracted using Soxhlet apparatus. 10gm of each sample was placed in thimble and extracted with diethyl ether which was boiled repeatedly for 7 cycles. The liquid leftover in a round bottom flask was used as oil extract while defatted sample in the thimble was used for phenolic determination. The total Phenolic content in each extract was determined by the Folin-Ciocalteu method [19] with slight changes. An equal amount of sample (1 mL) and Folin-Ciocalteu (1mL) reagent was incubated at room temperature for 3 min followed by the addition of 1 mL of saturated sodium carbonate solution (20%). The volume was adjusted to 10 mL with distilled water. The reaction mixture was kept in dark for 90 min, and absorbance was measured at 725 nm using UV-Visible spectrophotometer (Hitachi U-2900, Tokyo, Japan). A standard curve was generated by using different concentrations of gallic acid (50-500 mg) as standard and a blank was also prepared by using the same method.

2.3.3 Determination of Tannins

The tannin extraction was assessed with standard method described by Akindahunsi et al. 2006 [20]. Tannins were extracted by dissolving 0.5 gm of sample in 100 mL of 70% acetone. The solution was kept for 24 hours in shaking incubator followed by centrifugation, filtration and then stored at 4˚C. The stock solution of tannic acid was prepared by dissolving 50mg of tannic acid in 100mL of 70% acetone. Different concentrations of tannic acid (3mg – 50mg) were prepared by serial dilution from the stock solution (50 mg /100 mL 70% acetone), followed by the addition of 0.5 mL of Folin-phenol reagent and 2.5 mL of 20% Sodium carbonate (NaCO3). Blank containing 70% acetone was also prepared by using the same method. The absorbance was measured in triplicate using UV-Visible spectrophotometer (Hitachi U-2900, Tokyo, Japan).

2.4. Bioassays

2.4.1 Antioxidant activity

The antioxidant activity of crude extracts and phytochemicals was determined against stable 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) using modified method of Brand-Williams et al. 1995 [21]. The DPPH solution was made by dissolving 0.006gm of DPPH in 100 mL DMSO. 1 mL of each extract (15-1000 µg/mL) was mixed with 2 mL of DPPH solution. Positive control containing ascorbic acid (1-100 µg/mL) was also prepared. 1 mL methanol with 2 mL DPPH solution was prepared as a blank/ negative control. The absorbance was recorded at 517 nm using UV-Vis spectrophotometer (Hitachi U-2900, Tokyo, Japan). The dose response curve was generated to calculate IC_{50} values. The experiment was carried out in triplicates.

2.4.2 Antiglycation activity

A modified method of Nakagawa et al. (2002) [22] was used for antiglycation activity. In these experiments total volume of reaction was kept 1200 µL (400 µL bovine serum albumin (BSA) (10 mg/mL), 400 µL of glucose anhydrous (50 mg/mL) and 400 µL test sample). The composition of Glycated control was 400 µL BSA, 400 µL glucose and 400 µL sodium phosphate buffer, while blank control consisted of 400 µL BSA and 800 µL sodium phosphate buffer. The reaction mixture was then incubated at 37°C for 7 days. Followed incubation, 120 µL of trichloroacetic acid (TCA) was introduced and then centrifuged (15,000 rpm) for 4 min at 4°C. Next, the pellets obtained were washed using 1200 µL (10%) of TCA. The pellets contained the advance glycated end products (AGE)-BSA and were redissolved in 1200 µL phosphate buffer solution (PBS) while the supernatant containing glucose, inhibitor as well as interfering substances was discarded. Measurements of fluorescence spectrum (excitation 370 nm), and changes in fluorescence intensity (excitation 370 nm to emission 440 nm) based on AGEs were monitored by using spectroflouro-photometer (RF-5301 PC), Shimadzu Japan.

2.5 Statistical Analysis

The results were analyzed in Graph Pad Prism 5 using non-linear regression analysis. For the calculation of IC_{50} values, dose response curve was generated by plotting log inhibitor concentrations on X-axis and absorbance/fluorescence on Y-axis. All results were represented as mean ± standard error of mean (S.E.M).

3.0 Results for Bioactive compound determination

3.1 Results for Flavonoid determination

Total flavonoids were measured in all developed polyherbal formulations (n=11) as well as individual plants in the first set of trials. To create a standard curve, different concentrations of quercetin (7.81 g/mL to 1000 g/mL) were employed as a standard (Figure 1 A). Results revealed that formulation S28 (*Trillium govanianum* + *Centurium teniflorium* + *Marcheloconica* + *Robinia pseudoacacia* contained highest concentration of flavonoids (895.1±16.8 µg/mL) followed by the S27, S32, S31, S22, S23, S29, S30, S26, S24, and S25. The values were found to be 889.2±4, 836.9±39.7, 817.7±12.4, 681.5±17.7, 624.4±25.21, 620.4±23, 547.1±9.3, 477±47.5, 413.5±131.3, and 215 ±19.3 µg/mL respectively. When the individual plants were evaluated for flavonoids determination the highest concentration was shown by the S1 (*Trillium govanianum*) followed by the NP (*Rubinia pesduoacacia*) and S2 (*Centaurium tenuiflorum*) with concentrations of 699.3±12.6 µg/mL, 592.5±7.0 µg/mL and 339.6±2.3 µg/mL respectively.

3.2 Results for Phenol determination

In a second series of experiments, total phenols were determined in all developed polyherbal formulations (n=11) as well as individual plants. To generate a standard curve different concentrations (7.81–1000 g/mL) of gallic acid were used in graph pad prism (Figure 1B). The data revealed highest phenol concentration in formulation S28 (*Trillium govanianum + Centurium teniflorium + Marcheloconica + Robinia pseudoacacia* (929.0±1.9 µg/mL) followed by S32, S31, S29, S27, S23, S30, S22, S25, S26 and S24 (*Nigella sativum+morcheloconica*) respectively the values were found to be 779.9±11.6, 643.6±2.2, 610.3±13.8, 574.9±10.7, 569.3±36.4, 539.3±38.5, 523.0±17.1, 407.9±18.1, 393.6±1.2 and 328.4±1.4 µg/mL respectively. When the individual plants were evaluated for phenol quantification the S1 (*Trillium govanianum*) showed comparatively good concentration as compared to NP (*Rubinia pesduoacacia*) and S2 (*Centaurium tenuiflorum*). The values were found to be 624.2±31.2, 394.2±3.0 and 316.3±3.27 µg/mL respectively.

3.3 Results for Tannin determination

The tannic components for all polyherbal formulations as well as for individual plants were measured in a third series of experiments. A standard curve was generated in graph pad prism using various tannic acid concentrations (7.81g/mL to 1000g/mL) as shown in Figure 1C. The highest concentration of tannins was found in formulation S28 *(Trillium govanianum + Centurium teniflorium + Marcheloconica + Robinia pseudoacacia* followed by S22, S30, S32, S27, S31, S29, S23, S26, S24 and S25 respectively. The value was found to be 783.9±5.4, 711.6±12.1, 648.8±5.8, 633.8±12, 605.3±16.3, 552.4±10.1, 543.9±2.1, 521.7±10.7, 453.3±18, 414.3±12.2, and 350.2±2 µg/mL respectively. When the individual plants were checked for tannins determinations again S1 (*Trillium govanianum*) showed the highest concentration of tannins as compared to NP (*Robinia pseudoacacia* and S2 (*Centaurium tenuiflorum*) the values were found to be 498.8±5.8, 420.6±4.4, and 103.7±13.1 µg/mL respectively.

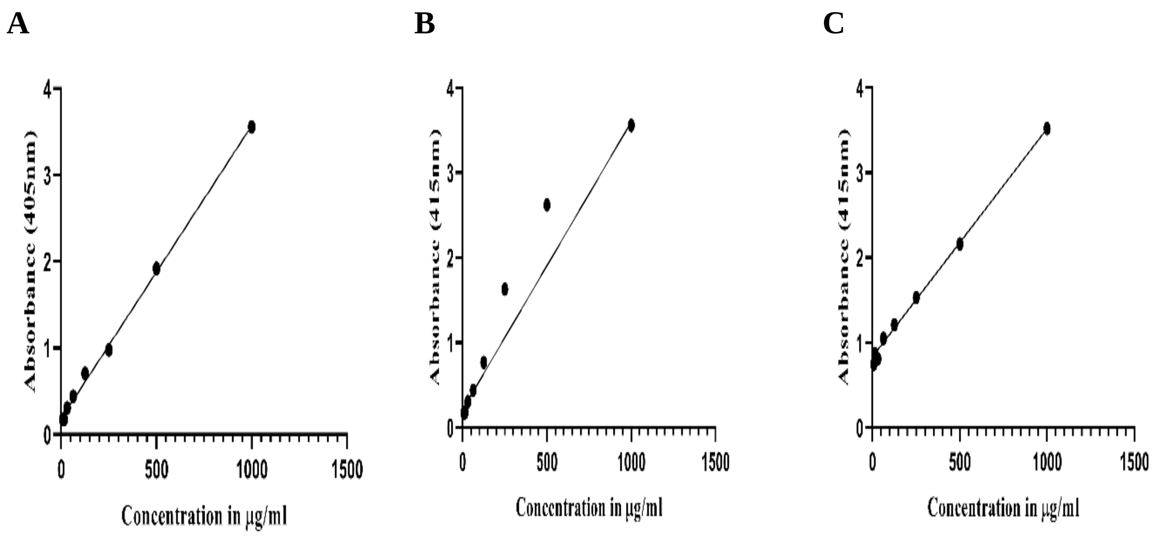

Figure 1: A graphical representation of phytochemical determination of polyherbal formulations and individual plants. (A) Standard curve for flavonoid determination using quercetin as standard. (B) Standard curve for phenols determination using gallic acid (C) Standard curve for tannins determination using tannic acid.

Table 2. Concentration of flavonoids, phenols, and tannins in different selected polyherbal formulation and individual plants. Results are shown as Mean ± SD

Samples	Flavonoids (µg/mL)	Phenols (µg/mL)	Tannins (µg/mL)
S1	699.3± 12.6	624.2±31.2	498.8±5.8
S2	339.6±2.3	316.3±3.27	103.7±13.1
NP	592.5±7.0	394.2±3.0	420.6±4.4
S22	681.5±17.7	523.0±17.1	711.6±12.1
S23	624.4±25.21	569.3±36.4	521.7±10.7
S24	413.5±131.3	328.4±1.4	414.3±12.2
S25	215.0 ±19.3	407.9±18.1	350.2±2.0
S26	477.0±47.5	393.6±1.2	453.3±18.0
S27	889.2±44.0	574.9±10.7	605.3±16.3
S28	895.1±16.8	929.0±1.9	783.9±5.4
S29	620.4±23.0	610.3± 13.8	543.9±2.1
S30	547.1 ±9.33	539.3±38.5	648.8±5.8
S31	817.7 ±12.4	643.6±2.2	552.4±10.1
S32	836.9 ±39.7	779.9±11.6	633.8±12.0

3.4 Bioassays
3.4.1 Antioxidant activity
The antioxidant activities of crude extract and phytochemicals including phenol, flavonoid and tannins were analyzed used the DPPH scavenging assay. To produce a dose response curve, a variety of concentrations have been assessed. The concentration of sample is directly proportional to the activity of that sample however a comparison was made based on IC_{50} values.

3.4.2 Antioxidant activity of crude extracts
The results for the crude extracts ranging from 21.63±0.15 µg/mL - 275.7±4.26µg/mL are shown in Table 3. The highest antioxidant activity is shown by the crude extract of formulation S22 *(Centaurium tenuiflorum + Morchela conica)* followed by S29, S28, S31, S26, S24, S23, S27, S30, S25 and *S32 (Centurium teniflorium + Robinia pseudoacacia* with IC_{50} values of 21.63±0.15, 31.35±0.38, 41.72±3.44, 48±1.54, 51.48±0.26, 63.29±0.15, 109.4±1.92, 116.3±1.76, 234.3±2.51, 246.9± 0.18 and 275.7±4.26µg/mL respectively. The least antioxidant activity was shown by *S32* (*Centurium teniflorium + Robinia pseudoacacia* with IC_{50} value of 275.7±4.26µg/mL. When the formulations were compared to their respective individual plants, most of the polyherbal formulations were found active in free radical scavenging assay, demonstrating the synergistic effect once more in polyherbal formulation. When the individual plants (S1), *Trillium govanianum*, (S2), *Centaurium tenuiflorum*, and (NP) *Rubiniapesduocacia* were evaluated for their antioxidant activities the most potent antioxidant activity was shown by (S2) *Centaurium tenuiflorum,* followed by (S1) *Trillium govanianum* and (NP) *Rubiniapesduocacia* with IC_{50} values of 23.7±0.01µg/mL, 68 ±0.02 µg/mL, 167.5±0.01µg/mL. The positive control (ascorbic acid) has showed the IC_{50} value of 20.81±2.01 µg/mL as shown in table 3. The overall comparison indicated the highest potential of polyherbal formulation S22 *(Centaurium tenuiflorum + Morchela conica)* among all formulations and individual plant (S2) *Centaurium tenuiflorum* among all other individual plants with IC_{50} values of 21.63±0.15 µg/mL and 23.7±0.01µg/mL respectively.

3.4.3 Antioxidant Activity of Flavonoids
The extracted flavonoids were also evaluated for the antioxidant potential. The results have been shown in table 3. The data indicated that antioxidant activities of polyherbal formulations ranged from 33.5±0.6µg/mL to 7909±12.1µg/mL. The highest antioxidant potential was found in formulation S24 (*Nigella sativum+ morchela conica*) followed by the S28, S22, S27, S30, S32, S31, S26, S25, S29, and S23 with IC_{50} values of 33.5±0.6, 40.61±0.56, 61.88±0.12, 79±4.32, 127.2±1.8, 136.3±0.78, 177.3±3.42, 288.6±0.33, 290.7±0.12, 371.2±0.25 and 711.3±3.06µg/mL respectively. When the antioxidant activities were compared for individual plants S1, S2 and NP it is noticed that the maximum antioxidant activity is found in (NP) *Rubiniapesduocacia* followed by (S1) *Trillium*

govanianum and (S2) *Centurium teniflorium* by showing the IC_{50} values of 35±0.13, 70±0.09 and 76.05±0.11µg/mL respectively. The data shows that the individual plant and polyherbal formulations have the highest antioxidant activities.

3.4.4 Antioxidant activity of Phenols

The results of antioxidant activity of phenols revealed IC_{50} values in a range of 7.6±0.36µg/mL to 1612.5±137.88 µg/mL. The highest antioxidant activity was shown by the formulation S28 (*Trillium govanianum* + *Centurium teniflorium* + *Marcheloconica* + *Robinia pseudoacacia* followed by S27, S31, S30, S25, S32, S29, S23, S22 and S24 with IC_{50} values of 7.6±0.36, 16.37±0.27, 17.07±0.99, 20.53 ±0.41, 50.62±3.6, 56.8±0.1, 63.59±3.6, 71.0 ±0.08, 156.7±0.84, 158±1.0 and 329.5±0.7µg/mL respectively. When the phenolic extracts of individual plants were compared it was noticed that the plant *Trillium govanianum* (S1, Phenols) shows comparatively good antioxidant activity among S2 and NP with IC_{50} values of 70.5±0.09, 76.9±0.08 and 166.6±0.23 µg/mL correspondingly. The sample were then compared with standard control (ascorbic acid, IC_{50} value of 20.81±2.01 µg/mL) the phenolics from S28, S27, and S31 showed the best antioxidant activities with IC_{50} values of 7.6±0.36, 16.37±0.27, and 20.53 ±0.4µg/mL respectively which were even higher than the individual plants thus give an indication of synergistic effects in polyherbal formulation for controlling oxidative stress.

3.4.5 Antioxidant activity of Tannins

The extracts derived from tannins were assessed for anti-oxidant activities and their results revealed that IC50 values in range from 8.17 ±1.69µg/mL to 260.8±5.37 µg/mL. The highest antioxidant activities were shown by the tannins extracted from S28 (*Trillium govanianum* + *Centurium teniflorium* + *Marcheloconica* + *Robinia pseudoacacia* followed by S27, S32, S22, S25, S24, S30, S23, S26, S31 and S29 with IC_{50} values of 8.17 ±1.69, 14.76±3.2, 36.29±3.13, 42.8±0.13, 56.0±0.10, 78.9±0.24, 101±3.27, 112.4±1.92, 116.7±0.10, 237±4.07, and 257±0.11µg/mL. When tannins from individual plants were evaluated for their antioxidant activities it was noticed that (S1) *Trillium govanianum* possess good antioxidant activity as compared to (S2) *Centurium teniflorium* and (NP) *Rubinia pesudoacacia* and with IC_{50} values of 69.9±0.6, 93.5±1.0 and 92.9±0.5µg/mL respectively. The samples were then compared with standard (ascorbic acid) havinf IC_{50} value of 20.81±2.01µg/mL its clear from above that the polyherbal formulations S28 and S27 have greater antioxidant activity than the individual plants indicating the synergistic effect of individual plants in polyherbal formulation.

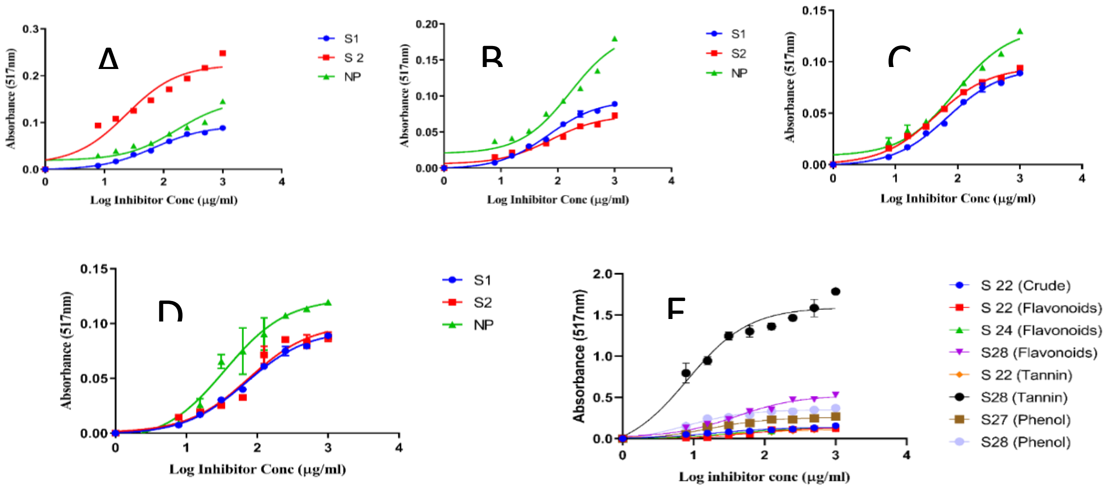

Figure 2: Dose response curves for antioxidant activities by individual plants and active polyherbal formulations at a concentration range of (7.8-1000 µg/mL). (A) Antioxidant activity of crude extract for individual plants. (B) Antioxidant activity of extracted phenols from individual plants. (C) Antioxidant activity of extracted tannins from individual plants. (D) Antioxidant activity of

extracted flavonoids from individual plants. (E) Antioxidant activity of potent crude extract and phytochemicals from polyherbal formulations.

Table 3. IC_{50} values for antioxidant properties of individual plants and polyherbal formulations

Plants/ Formulations	Crude Extract	Phenol	Flavonoids	Tannins
	IC_{50}±SE µg/ml			
S1	68.0±0.02	70.5±0.09	70±0.09	69.9±0.6
S2	23.7±0.01	76.9±0.08	76.05±0.11	93.5±1.0
NP	167.5±0.01	166.6±0.23	35±0.13	92.9±0.5
S22	21.63±0.15	158±1.0	61.88±0.12	42.8±0.13
S23	109.4±1.92	156.7±0.84	711.3±3.06	112.4±1.92
S24	63.29±0.15	329.5±0.7	33.5±0.6	78.9±0.24
S25	246.9± 0.18	56.8±0.1	290.7±0.12	56.0±0.10
S26	51.48±0.26	368.8 ±0.8	288.6±0.33	116.7±0.10
S27	116.3±1.76	16.37±0.27	79.0± 4.32	14.76±3.2
S28	41.72±3.44	7.6±0.36	40.61±0.56	8.17 ±1.69
S29	31.35±0.38	71.0 ±0.08	371.2±0.25	257±0.11
S30	234.3±2.51	50.62±3.6	127.2±1.80	101±3.27
S31	48.0±1.54	20.53 ±0.41	177.3±3.42	237±4.07
S32	275.7±4.26	63.59±3.6	136.3±0.78	36.29±3.13
Ascorbic acid	20.81±2.01			

3.5 Antiglycation activity

In another series of experiments antiglycation activity was carried out for individual plants as well as polyherbal formulations. In addition, the same has been evaluated for phenols, flavonoids and tannins extracted from individual plants as well as from formulations. Data indicated an increase in activity with increase in concentration till the maximum is achieved. Log inhibitor concentration was plotted on X-axis and fluorescence was plotted on Y-axis. Based on dose response curves IC_{50} values were calculated for comparison. All experiments were performed in duplicate with positive and negative controls.

3.5.1 Antiglycation activity of crude extract

Antiglycation activity of the crude extracts were conducted. The results of this activity are presented in Figure 3 & Table 4 which showed that the antiglycation activity of crude polyherbal formulation range from 6.53±2.09 to 86.63±1.91µg/mL. The highest antiglycation potential was found in crude extract of polyherbal formulation S25 (*Nigella sativum +Morchella conica*) followed by S24, S26, S29, S22, S23, S30, S28, S27, S32, and S31 with IC_{50} values of 6.53±2.09, 6.90±1.63, 9.10±1.79, 10.46±1.74, 10.63±1.72, 16.78±2.28, 29.65±2.08, 34.56±1.68, 49.45±1.48, 58.07±1.59 and 86.63±1.91µg/mL respectively. When the antiglycation activity of individual plants were evaluated sample (NP) *Rubiniapesduocacia* was found with the lowest IC_{50} value 15.18±1.05 followed by the (S1) *Trillium govanianum* and (S2) *Centaurium tenuiflorum* with IC_{50} values of 17.72±1.04, 38.90±1.08µg/mL respectively. It was found that most of the samples had potent antiglycation activities as compared to positive control rutin having IC_{50} value 22.5±0.9 µg/mL as shown in the table 4.

3.5.2 Antiglycation activity of Flavonoids

The extracted flavonoids were assessed for antiglycation activity as shown in table 4. The data reveal variable IC_{50} values ranging from 6.613±1.53 to 488.3±6.93 µg/mL. The highest antiglycation activity was shown by polyherbal formulation S30 (*Centaurium tenuiflorum + Robinia pseudo acacia*) followed by S29, S25, S24, S32, S22, S26, S31, S23, S27, and S28 with IC_{50} values of 6.613±1.53, 8.227±3.01, 8.210±1.53, 8.528±1.36, 11.96±1.67, 12.05±1.50, 12.17±1.79, 12.85±2.17, 26.96±1.98, 37.40±1.12, and 488.3±6.93µg/mL respectively. When the individual plants were evaluated for antiglycation activity it was observed that again (NP) *Rubinia pesduocacia* highest antiglycation activity with IC_{50} values of 15.73±1.19µg/mL followed by (S1) *Trillium govanianum* and (S2)

Centurium teniflorium with IC_{50} values of 34.73±0.95, 83.81±1.20 µg/mL respectively. When the polyherbal formulations were compared with rutin (IC_{50} value of 22.5±0.9µg/mL) it was noticed that formulations S30, S29, S25, S24, S32, S22, S26, and S31, are more active as compared to standard rutin and other polyherbal formulations with IC_{50} values of 6.613±1.53, 8.227±3.01, 8.210±1.53, 8.528±1.36, 11.96±1.67, 12.05±1.50, 12.17±1.79, 12.85±2.17µg/mL respectively.

3.5.3 Antiglycation activity of Phenols

The results of antiglycation activity of Phenols as shown in table 4 showed a variable range of IC_{50} values from 4.976±1.98 to 43.68±1.11 µg/mL. The highest antiglycation activity was shown by the phenolic extracts of polyherbal formulation S31 (*Centaurium tenuiflorum* + *Robinia pseudoacacia)* followed by S29, S24, S28, S25, S26, S22, S23, S32, S27 and S30 with the IC_{50} values of 4.976±1.98, 5.341±1.86, 6.766±1.51, 7.046±3.19, 7.124±1.69, 9.750±1.58, 10.28±1.40, 19.58±2.09, 30.83±2.04, 35.40±1.14 and 43.68±1.11µg/mL respectively. When the comparison was made for individual plants, it was noticed that (NP) *Rubiniapesduocacia* possess the lowest IC_{50} value of 27.38±1.18 µg/mL followed by (S1) *Trillium govanianum* and (S2) *Centaurium tenuiflorum* with IC_{50} values of 28.37±0.77 and 62.87±1.221 µg/mL correspondingly. When the samples were compared with positive control (rutin, IC_{50} value of 22.5±0.9µg/mL) the formulation S29, S24, S28, S25, S26, S22, S23 were found potent with IC_{50} values of 4.976±1.98, 5.341±1.86, 6.766±1.51, 7.046±3.19, 7.124±1.69, 9.750±1.58, 10.28±1.40, 19.58±2.09µg/mL respectively as shown in the table 4.

3.5.4 Antiglycation activity of Tannins

The tannins extracted from polyherbal formulations and individual plants were evaluated for their antiglycation activity. The data in table 4 revealed the antiglycation activity ranging from 7.338±2.29 to 78.20±1.31µg/mL. The highest antiglycation activity is shown by the polyherbal formulation S28 (*Trillium govanianum* + *Centaurium tenuiflorum* + *Morchella conica* + *Robinia pseudo acacia*) followed by S22, S31, S26, S24, S23, S30, S32, S25, S29 and S27 with IC_{50} values of 7.338±2.29, 10.19±1.22, 10.55±2.55, 12.53±1.46, 14.22±1.49, 20.19±1.82, 27.67±2.01, 33.69±2.27, 47.65±1.86, 58.07±1.59, and 78.20±1.31µg/m L respectively. when the individuals' plants extracted tannins were evaluated for their antiglycation activities it was noticed that (NP) *Rubinia pseudoacacia* followed by (S1) *Trillium govanianum* and (S2) *Centaurium tenuiflorum* showed good antiglycation activity with IC_{50} values of 24.92 ±1.22, 29.56±0.78, and 87.43±0.92µg/mL respectively. The polyherbal formulations (S28, S22, S31, S26, S24, and S23) showed comparatively potent antiglycation activities than the positive control (Rutin, 22.5±0.9µg/mL).

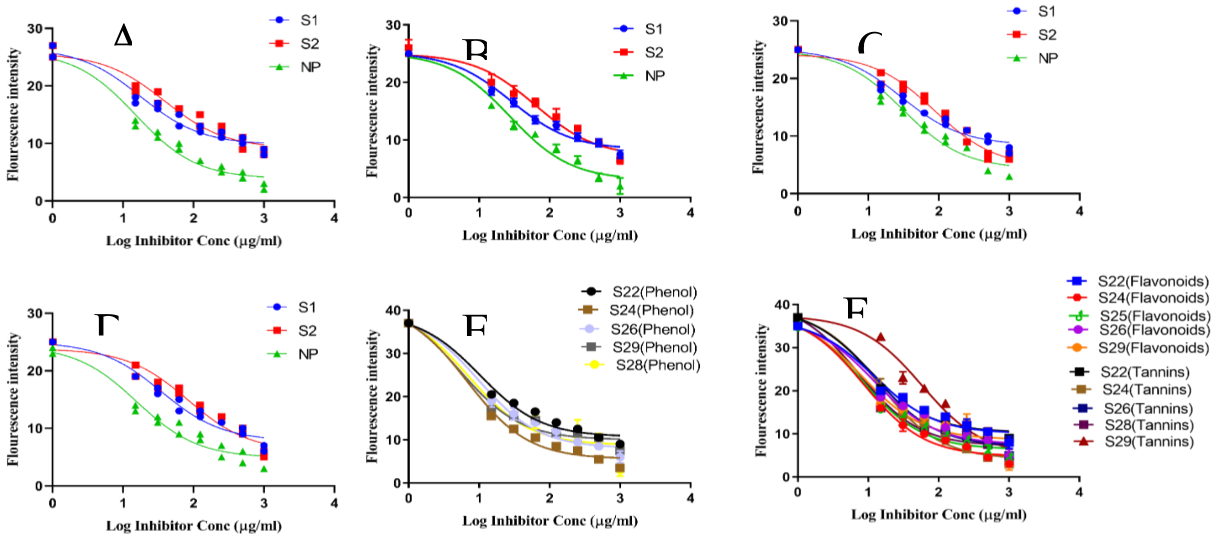

Figure 3: Dose response curves for antiglycation activity by individual plants and active polyherbal formulations at a concentration range of (7.8-1000 µg/mL). (A) Antiglycation activity of crude extract for individual plants. (B) Antiglycation activity of extracted phenols from individual plants.

(C) Antiglycation activity of extracted tannins from individual plants. (D) Antiglycation activity of extracted flavonoids from individual plants. (E&F) Antiglycation activity of potent crude extract and phytochemicals from polyherbal formulations.

Table 4: IC_{50} values for antiglycation potential of individual plants and polyherbal formulations.

Plants/ Formulations	**Crude Extract**	**Phenol**	**Flavonoids**	**Tannins**
	IC_{50}±SE μg/mL			
S1	17.72±1.04	28.37±0.77	34.73±0.95	29.56±0.78
S2	38.90± 1.08	62.87±1.221	83.81±1.20	87.43±0.92
NP	15.18±1.05	27.38±1.18	15.73±1.19	24.92 ±1.22
S22	10.63±1.72	10.28±1.40	12.05±1.50	10.19±1.22
S23	16.78±2.28	19.58±2.09	26.96±1.98	20.19±1.82
S24	6.90±1.63	6.766±1.51	8.528±1.36	14.22±1.49
S25	6.53±2.09	7.124±1.69	8.210±1.53	47.65±1.86
S26	9.10±1.79	9.750±1.58	12.17±1.79	12.53±1.46
S27	49.45±1.48	35.40±1.14	37.40±1.12	78.20±1.31
S28	34.56±1.68	7.046±3.19	488.3±6.93	7.338±2.29
S29	10.46±1.74	5.341±1.86	8.227±3.01	58.07±1.59
S30	29.65±2.08	43.68±1.11	6.613±1.53	27.67±2.01
S31	86.63±1.91	4.976±1.98	12.85±2.17	10.55±2.55
S32	58.07±1.59	30.83±2.04	11.96±1.67	33.69±2.27
Rutin	22.5±0.9			

4.0 DISCUSSION

Diabetes mellitus is an endocrinological disorder that arises due to impaired insulin secretion, insulin action or production in the body [23]. Several synthetic antidiabetic drugs as target-inhibitors are available but they pose several limitations, and still there is a growing interest of new potent inhibitors from natural sources that may overcome the adverse effects of existing drugs [19]. In the current study antioxidant and antiglycation activity of three individual plants and polyherbal formulations (n =11) were evaluated. Moreover, phytochemicals including phenols, flavonoids and tannins were also quantified and assessed for their bioactivity. The studied plants were selected using ethnomedicinal approach and consequently formulations were made by the mixing individual plants *Trillidium govanianum* (S1), *Centaurium tenuiflorum* (S2), *Cichorium intybus, Trigonella caerulea, Saussurea lappa, Lepidium sativum, Nigella sativa,Morchella conica* (S10) and *Rubinia pseudoacacia* (NP) plant . Among these plants, S1, S2, NP, have also been studied individually in addition to their polyherbal formulations. While the rest of the plants have individually been studied previously by our group [19]. All the developed polyherbal formulation and plants were prepared and characterized for their inhibitory potential against, AGEs, and antioxidant activities to find a potential multi-targeted inhibitor. The accumulation of AGEs plays a primary role in diabetes complications. Polyherbal formulation provide important path for novel antidiabetic agents (Telapolu, Kalachavedu, Punnoose, Bilikere, & medicine, 2018). The antioxidant and antiglycation activities were compared and it is noticed that various phytochemicals extracted form polyherbal formulation S28 showed potent activity including phenols, flavonoids, and tannins. Where phenols and tannins from S28 showed highest free radical scavenging activity by showing IC_{50} values of 7.6±0.36μg/mL and 8.17±1.69μg/mL respectively. While phenols and tannins were found to be active in antiglycation assay. The IC_{50} values were found to be 7.046±3.19μg/mL and 7.338±2.29μg/mL respectively. In addition, comparison of crude extracts showed the potency of polyherbal formulation S22 (*Centorium tenuiflorum+Morchella conica*) by showing IC_{50} values of 21.63±0.15μg/mL and 10.63±1.72μg/mL in antioxidant and antiglycation assays respectively. The results are found comparable to ascorbic acid (20.81±2.01) and rutin (22.5±0.9μg/mL) which were taken as a standard for antioxidant and antiglycation activities respectively.All other polyherbal formulations showed comparable antiglycation activity to each other except S27 and S32 that showed higher IC_{50} values thus lower activity. AGEs in correlation to oxidative stress is mainly responsible for diabetic complications [24].

Antioxidants present in polyherbal formulations could be an effective tool to prevent diabetes as they have the capacity to stabilize the free radicals and have the potential to control the AGEs. In this regard polyherbal formulations provide an important path for novel antidiabetic agents [25 & 26]. Fernando et al. [27] studied the effect of aqueous extracts of *Nawarathne kalka* against AGEs using fluorescence spectroscopic method [27]. Their data indicated potency of extract by showing IC_{50} values of 84±28 µg/mL [24].

Our results were found consistent with the literature by showing potent antioxidant and antiglycation activity by polyherbal formulations S22, S23, S24, and S26 (crude, phenols, tannins, and flavonoids), S28 (phenols and tannins) and S29 (flavonoids, phenols) among all the studied formulations. The overall data comparison reveals the potency of polyherbal formulations as compared to individual plants thus showing the synergistic behavior in formulation. Gupta et al. [28] evaluated antioxidant activity of polyherbal formulation comprises of stem bark of *Ficus glomerata Roxb* and *Symplocos racemosa Roxb* using DPPH scavenging assay [28]. The IC_{50} values were found to be 41.529 µg/mL, 39.654 µg/mL and 17.511 µg/mL for total phenolic, aqueous extract and ascorbic acid respectively. Our results are also found consistent with this one by showing potential antioxidant and antiglycation properties by S28. Additionally, our study also reports the amylase and glucosidase inhibitory potential of polyherbal formulations S27 and S28.

When the data were compared for phenols and flavonoids contents it is noticed that S28 (*Trillidium goanum+Centorium tenuiflorum+Morchella conica+Rubinia pseudoacacia*) possess highest concentration of flavonoids, phenols and tannins (895.1±168µg/mL., 929.0±1.98µg/mL, 783.9±5.48µg/mL) followed by S27 (889.2±44 µg/mL, 574±10.7µg/mL, 605.3±16.3µg/mL) respectively as compared to individual plants and other formulations. It has been indicated several times in literature that most of the activity in plants is due to flavonoids as these are polyphenols with many hydroxyl groups [24]. The importance of flavonoids had also been shown from French studies [29]. Reported that French people used red wine that had higher amounts of flavonoids that's why they got less chance of cardiovascular diseases. In addition flavonoid contents have already been quantified in different polyherbal formulation but data related to bioactivity were limited. Aslam et al. [30] evaluated phytochemical contents in herbal formulation of *C. nutans* and *E. scaber*. Higher content of flavonoids was found in formulation as compared to individual plant *E. scaber*. Our results are in line with these studies by showing higher amounts of flavonoids in polyherbal formulations S27 and S28 in comparison to individual plants, phenol, and tannins. Additionally, the current polyherbal formulation showed a synergistic effect in the Antiglycation assay as well as antioxidant assays. Therefore, these formulations can be considered as multi-targeted inhibitors for antidiabetic drug development process and have been selected for in vivo experiments in addition to individual plants (S1, S2 and NP). In conclusion this study showed the potent polyherbal formulations S27, S28 against amylase, glucosidase, and AGEs as compared to other formulations as well as individual plants thus showed the synergistic behavior of plants when used in combination with each other. These activities can be correlated with phytochemicals including flavonoids and phenols.

Study conducted by Raphael [31] an activity on the polyherbal formulation of the stem bark of *Ficus glomerata Roxb* and *Symplocos racemosa Roxb* in which evaluate the antioxidant activity on DPPH method [31]. The IC_{50} value of total phenolic, aqueous extract and ascorbic acid was found 41.529 µg/mL, 39.654 µg/mL and 17.511 µg/mL respectively which show weak inhibition than present study sample S22 of crude, S28 of phenol, S24 of flavonoid and again S28 of tannins was found good antioxidant activity, with IC_{50} values of 21.63±0.15, 7.6±0.36, 33.5±0.6 and 8.17 ±1.69 respectively. The current study also showed the synergistic behavior of this formulation towards AGEs and oxidative stress indicating that this formulation without addition of any synthetic compounds can directly control diabetes the complications by controlling AGEs and reactive oxygen species. These activities can be correlated with phytochemicals including flavonoids and phenols [32]. It has already been reported in literature that flavonoids are mostly polyphenols which provide a major contribution towards bioactivity due to the presence of several hydroxyl groups. Flavonoids gained much attention since the discovery of the French paradox. The French people got less chance of heart attack due to increased use of red wine which contained higher number of flavonoids [29]. Several groups have already studied the flavonoids contents in polyherbal formulation, but lack the data related to bioactivity of those flavonoids. The phytochemical evaluation of polyherbal formulation of *C. nutans* and *E. scaber* and found out higher contents of flavonoids in *C. nutans* and formulation of two plants as compared to individual plant *E. scaber* [30]. Our results are found to be consistent with this study by reporting the higher contents of flavonoids in polyherbal formulations as compared to simple phenols as well as individual plants. Additionally, the current polyherbal formulation showed a synergistic effect in the Antiglycation assay as well as antioxidant assays**.** In conclusion polyherbal formulations S22, S28, S29 and S24 formulations reported in this study can be used as multi targeted inhibitors to treat Diabetes mellitus as it became active in antiglycation assays and antioxidant assays as well. However, DIT needs to be calculated and further in vivo assays are required for proof of concept. Secondly, the activity might be due to the flavonoids, which were found in higher concentration as compared to phenols. The data clearly demonstrates the synergistic effect of individual plants in formulation, thus indicating that these can be further used as multi-targeted antidiabetic drug candidate. However, further in vivo anti-diabetic analysis of S22, S28, and S24 polyherbal formulations and their individual plants S1, S2, and NP is under progress.

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Authors' Contributions
TM and SF performed the experiments, collected the plants, and wrote the first draft of manuscript. NB prepared the polyherbal formulations and collected plants. ZP designed the study, performed the statistical analysis, helped in experiments and manuscript writing. BK, MA and AWQ reviewed the manuscript and carried out the statistical analysis. JH and AM helped in proof reading of the final draft and scientific discussions.

Competing Interests. The authors declare that there is no conflict of interest.

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