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PRELIMINARY PHYTOCHEMICAL INVESTIGATION AND HPTLC FINGER PRINTING OF AN ANTIDIABETIC POLYHERBAL FORMULATION

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ABSTRACT: The aim of the present work was to investigate the phytochemical components of an antidiabetic polyherbal formulation. The formulation was prepared using ingredients of *Azadirachta indica* A. Juss, *Commiphora mukul*, *Curcuma longa* L, *Emblica officinalis* Gaertn, *Garcinia cambogia* Gaertn, *Gymnema sylvestre* Retz, *Momordica charantia* L, *Ocimum sanctum* L and *Trigonella foenum-graecum* L. The formulation was evaluated for phytochemical properties by following standard protocols. The colour intensity or the precipitate formation was used as analytical responses to these tests. The formulation revealed the presence of active phytoconstituents such as carbohydrates, amino acid, proteins, phenols, flavonoids, tannins, terpenoids, steroids and insulin. The HPTLC fingerprint analysis revealed sixteen bioactive compounds when scanned at 520 nm and thirteen bioactive compounds at 254 nm. The study confirms that the formulation contains many phytoconstituents making it a prospective candidate for further development

INTRODUCTION: Medicinal plants have been found to have wide therapeutic application against various ailments. The phytochemicals present in the plants are responsible for their therapeutic properties. Phytochemicals are also responsible for different colour, flavour and smell of plants. Natural products such as pure phytoconstituents, polyherbal formulations and plant extracts offer huge opportunities for new drug development due to their unparalleled chemical diversity. Phytochemical analysis of plants which were used in folklore has yielded a number of active compounds with various pharmacological activities.

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The modern methods describing the identification of active phytoconstituents in the plant material may be useful for proper standardization of herbal drugs by HPTLC technique [1, 2]. Currently, HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity [3].

The beneficial effects of various Indian herbs are reported by various researchers. The chemo preventive effect of ethanolic extract of neem leaf against N-methyl-N`-nitro-N-nitrosoguanidine (MNNG)-induced oxidative stress has been reported[4]. Treatment with petroleum ether extract of kernel and husk of neem seeds exhibited protection against oxidative stress in heart and erythrocytes in streptozocin-diabetic animals [5]. Bhat et al reported that the chloroform and methanolic leaf extract of *Azardiract indica* significantly increased the glucose-6-phosphate

dehydrogenase activity in hepatic cells, plasma insulin and c-peptide levels after 21 days of treatment in mice [6]. The active ingredients of *Commiphora mukul* have excellent cholesterol lowering property [7]. Shishodia et al reported that guggulsterone inhibits the proliferation of wide variety of human tumour cell types including leukaemia, head and neck carcinoma, multiple myeloma, lung carcinoma, melanoma, breast carcinoma, and ovarian carcinoma [8]. Curcumin and its derivatives extracted from the rhizomes of the *Curcuma longa* L. have been reported to possess hypoglycemic effects on genetically diabetic KK-Ay mice [9]. Orally ingested curcumin reversed obesity-associated inflammation in diabetic mice [10]. The aqueous seed extract of *Emblica officinalis* Gaertn showed anti-diabetic activity in diabetic rats [11]. Another study reported the antidiabetic and antioxidant potential of *Emblica officinalis* Gaertn leaf extract in streptozotocin-induced type-2 diabetes mellitus (T2DM) in rats [12]. D'souza et al reported the active constituents and modes of action of the Indian indigenous fruit *Emblica officinalis* Gaertn against diabetes [13]. Hydroxycitrate, the active constituent of *Garcinia cambogia* Gaertn, is reported to enhance glycogen synthesis in exercised human skeletal muscle [14].

The protective effect of Garcinia against renal oxidative stress and biomarkers induced by high fat and sucrose diet has been studied [15]. Gymnemic acid extracted from *Gymnema sylvestre* is reported to have antidiabetic and antioxidant activity[16], anti-obesity and cardioprotective effect [17] in animal models. Methanolic fruit extract of *Momordica charantia* L showed hypoglycemic effect in diabetic rats [18]. Singh et al reviewed the active constituents and modes of actions of the anti-diabetic effects of *Momordica charantia* [19]. Hypercholesterolemia-induced erythrocyte lipid peroxidation activity was inhibited by aqueous extract of *Ocimum sanctum* L in male albino rabbits [20]. Another study reported that the seed oil of *O.sanctum* possess antidiabetic, antihypercholesterolaemic and antioxidant effects [21]. The wound healing activity of *Ocimum sanctum* L with supportive role of antioxidant enzymes has been reported by Shetty et al [22]. The oral administration of methanolic extract of fenugreek resulted in hypoglycaemic effect in mice [23]. Administration of *Trigonella foenum-graecum* (fenugreek) seed extract also showed lower blood glucose, glycated hemoglobin, triglycerides and total cholesterol in streptozotocin-induced diabetic rats [24].

The aim of our present study was to evaluate the phytoconstituents of a novel antidiabetic polyherbal formulation formulation that was prepared using a patented aqueous extraction procedure. The use of aqueous extraction procedure eliminates the problem associated with residual solvents present in the finished product and reduces the cost of production, considerably.

MATERIALS AND METHODS:

Plant Materials: The polyherbal formulation was gifted by M/s. Lanson Biotech, Chennai. The list of plants used for the preparation of the formulation and the percentage of individual plant in the formulation are provided in **Table 1**.

Extraction of the plant materials:

The formulation was prepared using a patented aqueous extraction procedure [25]. The herbs were collected from organic farms, which were free from pesticide residue. The herbs were cleaned in natural mineral water. Required quantities (as mentioned in **Table 1)** of fruit pulp with seed of *Momordica charantia* L, fruit pulp of *Emblica officinalis* Gaertn and leaves of *Gymnema sylvestre* Retz were extracted by pounding without any application of heat at room temperature and fresh juice was obtained.

The outer coating of *Azadirachat indica* A. Juss seed kernal were removed and was extracted using rotary press, without any external heating. The seeds of *Trigonella foenum-graecum* L, rhizome of *Curcuma longa* L, fruits of *Garcinia cambogia* Gaertn and resins of *Commiphora mukul*, Leaves & seeds of *Ocimum sanctum* L were air dried at room temperature and made a fine powder using a mixer. The mixture of fine powder obtained was soaked in the juice obtained from the other plants, which acted as a natural organic solvent. The extract was dried at room temperature without any heating process.

TABLE1. INGREDIENTS OF THE ANTIDIABETIC POLYHERBAL FORMULATION

S. No.	Botanical Name	English Name	Hindi Name	Tamil Name	Family	% Used	Used part
1.	*Azadirachta indica* A. Juss.	Neem	Neem	Vemmu	Meliaceae	10	Seed kernel
2.	*Commiphora mukul*	Indian Bdellium	Guggul	Erumaikan	Burseraceae	1	Resin – guggulsterone
3.	*Curcuma longa* L.	Turmeric	Haldi	Manjal	Zingiberaceae	10	Rhizomes
4.	*Emblica officinalis* Gaertn.	Indian Gooseberry	Amla	Nellikkai	Euphorbiaceae	20	Fruit pulp
5.	*Garcinia cambogia* Gaertn.	Gamboge	Goraka	Kodakapuli	Clusiaceae	1	Fruit pulp
6.	*Gymnema sylvestre* Retz.	Periploca of the woods	Gurmar	Cherukurinja	Asclepiadaceae	20	Leaves
7.	*Momordica charantia* L.	Bitter gourd	Karela	Pagakkai	Cucurbitaceae	20	Fruit pulp with seeds
8.	*Ocimum sanctum* L.	Holy Basil	Tulsi	Thulasi	Lamiaceae	8	Leaves & seeds
9.	*Trigonellafoenum-graecum* L.	Fenugreek	Methi	Vendhayam	Fabaceae	10	Seeds

Chemicals and reagents:
The chemicals and reagents used in the study were of analytical grade.

Phytochemical investigation:
The phytoconstituents of the antidiabetic polyherbal formulation were studied using standard procedures as described by Kokate [26]. The formation of colour or precipitate, depending on the end point of the respective test, was noted after the addition of the reagent and the outcome of the test was represented as present (+) or absent (-). All the tests except those required the powder form of the formulation were carried out with freshly prepared stock solution of the formulation with a concentration of 1mg/mL.

Test for carbohydrates:
a. Molisch's Test:
To one millilitre test solution was added a few drops of Molisch's reagent (5% alcoholic α-naphthol) followed by 2 mL of concentrated sulphuric acid along the inner side of the test tube.

b. Benedict's test for reducing sugar:
To one millilitre of test solution was added 5 mL of Benedict's reagent and kept boiling in a water bath for 5-7 minutes.

c. Fehling's test for reducing sugar:
To two millilitres of prior mixed equal volume Fehling's solution A and B was added 1mL of test solution and kept in boiling water bath for 5-10 minutes.

d. Selivanoff's test for ketones sugar:
To one millilitre of test solution was added 1mL of Selivanoff's reagent and 1mL of concentrated hydrochloric acid followed and kept in boiling water bath for 4-8 minutes.

Test for amino acid:
To one millilitre of test solution was added a few drops of 0.25% of ninhydrin reagent and kept in a boiling water bath for 2-5 minutes.

Test for proteins:
Heat method:
One millilitre of test solution was taken in a test tube and heated for 5-10 minutes using a water bath.

Biuret test:
To one millilitre of test solution was added 1 mL of Biuret reagent.

Test for Phenols:
To one millilitre test solution was added 1 mL of Folin-Ciocalteu reagent and 0.5 ml of Na_2CO_3.

Test for Flavonoids:
a. Shinoda Test:
To one millilitre of test solution were added fragments of magnesium ribbon and a few drops of concentrated hydrochloric acid.

b. **Zinc-Hydrochloride test:**
To one millilitre of test solution were added zinc dust and a few drops of hydrochloric acid.

Test for Tannins:
To one millilitre of test solution was added a few drops of 5% ferric chloride.

Test for Terpenoids:
To five millilitres of test solution was added 2 mL of chloroform and 3 mL of concentrated sulphuric acid.

Test for Steroids:
a. Sulphur powder test:
To one millilitre of test solution was added a little amount of sulphur powder and mixed well.

b. **Salkowski's test:**
To one millilitre of test solution was added a few drops of sulphuric acid and shaken well.

c. LibermanBurchard's test:
To one millilitre of test solution were added a few drops of acetic anhydride, boiled in boiling water for 3-5 minutes and after cooling added 1 ml of concentrated sulphuric acid.

Test for Glycosides:
General test:
Test A: Two hundred milligram of the formulation was mixed with 5mL of dilute sulphuric acid by warming in a water bath, filtered, and neutralized by adding of 5% of sodium hydroxide solution until it became alkaline (tested with pH paper). Added 0.1 mL of Fehling's solution A and B and heated in a water bath for 2 minutes.

Test B: Repeated 'Test A, procedure by using 5mL of water instead of dilute sulphuric acid. Compared whether the intensity of precipitation showed in Test A was more than Test B.

Test for Saponins:
Froth test:
The test solution (2mL) was shaken well in test tube and observed for froth (foam) formation.

Test for Anthocynidine:
a. To two millilitre of test solution was added concentrated H_2SO_4.

b. To two millilitre of test solution was added NaOH.

Test for insulin:
To one millilitre of test solution was added the solution of α-naphthol and sulphuric acid.

HPTLC (High Performance Thin Layer Chromatography) Fingerprint analysis:
The HPTLC fingerprinting of the polyherbal formulation was carried out at Captain Srinivasa Murti Research Institute of Ayurveda and Siddha Drug Development, Arumbakkam, Chennai-600106. The formulation (2.0gm) was taken in a 100mL conical flask, mixed with water: ethyl acetate (1:1), shaken well and transferred to a 250 ml separating funnel. After shaking, the upper layer (ethyl acetate) was collected. The aqueous layer was repeatedly (thrice) shaken with ethyl acetate. The ethyl acetate fraction was filtered and dried over anhydrous sodium sulphate and concentrated up to 10mL.

Ten millilitre and fifteen millilitre of the extract were applied in individual tracks on a Merck aluminium plate precoated with silica gel $60F_{254}$ of 0.2 mm thickness and the plate was developed in toluene: ethyl acetate (4:1) solvent system. The plate was visualised under UV 254 nm and 366 nm and photo documentation was done. The plate was scanned at 254 nm wavelength. Thereafter the plate was dipped in vanillin sulphuric acid and heated at 105˚C till the colour of the spots appeared and photo documentation was done under white light. The plate was then scanned at 520 nm wavelength.

RESULTS AND DISCUSSION:
Investigation of the aqueous extract of the antidiabetic polyherbal formulation revealed the presence of carbohydrates, amino acid, proteins, phenols, flavonoids, tannins, terpenoids, steroids and insulin whereas glycosides, saponins and anthocynidine were absent in the formulation as mentioned in **Table 2.** Phenol and flavonoid are strong antioxidants and are associated with many useful biological effects [27]. It is well established that phenolic rich diet and beverages may increase plasma antioxidant capacity. Polyphenol rich diets provide significant protection against progressive cellular damage of many chronic pathological

conditions including cancer, diabetes, cardio-vascular problems and aging [28]. Robert et al reviewed and reported that the therapeutic potential of flavonoids is due to their antioxidative, anti-atherosclerotic, antitumor and anti-inflammatory effects [29]. Richelle et al reported that tannins have antioxidative property which is important in protecting cellular oxidative damage including lipid peroxidation whereas plant sterols reduce cholesterol absorption, which leads to a decrease in LDL-cholesterol concentrations in plasma [30].

TABLE 2: RESULTS OF QUALITATIVE INVESTIGATION OF THE ANTIDIABETICPOLYHERBAL FORMULATION

S. No.	Phyto-constituent	Name of the test	Observation	Grading of observations
1.	Carbohydrates	Molisch's Test	Red violet ring appeared at the junction of two reagents	+
		Benedict's test	Reddish colour	+
		Fehling's test	Brownish red precipitation	+
		Selivanoff's test	No rose colour	-
2.	Amino acid	Ninhydrin test	Blue colour	++
3.	Proteins	Heat method	Coagulation formed	++
		Biuret test	Violet colour	++
4.	Phenols	Folin-Ciocalteu test	Blue colour	+++
5.	Flavonoids	Shinoda Test	Green to blue colour	+++
		Zinc-Hydrochloride test	Red colour	+++
6.	Tannins	Ferric chloride test	Green colour	++
7.	Terpenoids	Salkowski's test	Reddish brown precipitate	++
8.	Steroids	Sulphur powder test	The Sulphur powder sank to the bottom	++
		Salkowski test	Red colour at lower layer	++
		LibermanBurchard'stest	Green colour at upper layer	++
9.	Glycosides	General test	No red precipitation	-
10.	Saponins	Froth test	No froth formation	-
11.	Anthocynidine	H_2SO_4 test	No yellow or orange colour	-
		NaOH test	No blue colour	-
12.	Test for insulin	α -Naphtholand sulphuric acid test	Brownish red colour	++

Note; +++ = Appreciable amount; ++ = Moderate amount; + = Trace amount; - = Absent

Finger printing analysis of the formulation was carried out using the HPTLC method. The results of the analysis are summarised in **Table 3 & 4** and **Fig.1-3**. The scanning done at wavelength 520 nm showed a total of sixteen bioactive compounds with R_f values 0.04, 0.09, 0.13, 0.15, 0.29, 0.38, 0.47, 0.54, 0.60, 0.66, 0.69, 0.75, 0.82, 0.84, 0.88 and 0.94 with percentage peak area 0.44, 0.58, 1.38, 0.33, 20.15, 3.99, 5.60, 5.23, 15.22, 1.07, 0.47, 1.77, 5.57, 17.96, 6.22 and 14.04, respectively. Out of the sixteen compounds, the compounds with R_f values 0.18, 0.42, 0.50, 0.56, 0.80, 0.83, 0.88 and 0.92 were found to be predominant with peak area percentage in the range of 5.23 to 20.15. The rest of the compounds may be considered as insignificant since their peak area percentage was in the range of 0.33 to 3.99. The maximum peak area percentage observed was 20.15 for the compound with the R_f value of 0.18. The scanning of plate at 254nm revealed thirteen bioactive compounds with R_f values 0.04, 0.09, 0.17, 0.25, 0.30, 0.40, 0.48, 0.53, 0.50, 0.56, 0.74, 0.84 and 0.92 with peak area percentage 0.71, 0.90, 0.40, 13.83, 22.01, 10.65, 2.40, 0.36, 6.29, 1.09, 1.45, 26.38 and 13.53, respectively. Among these thirteen compounds, the compounds with R_f values 0.19, 0.27, 0.37, 0.57, 0.77 and 0.89 were the major compounds with a peak area percentage in the range of 6.29 to 26.38. The remaining compounds showed insignificant peak area percentage (less than 2.40). The maximum peak area percentage observed was 26.38 for the compound with the R_f value of 0.77.

Demands of herbal medicines have increased in the last two decades. So there is a need to develop new tests and bioassays to ensure the quality, safety and efficacy of herbal drugs[31]. Phytochemical standardization is one of the tools for the quality assessment of herbal drugs, which includes preliminary phytochemical screening and HPTLC fingerprint analysis using modern analytical

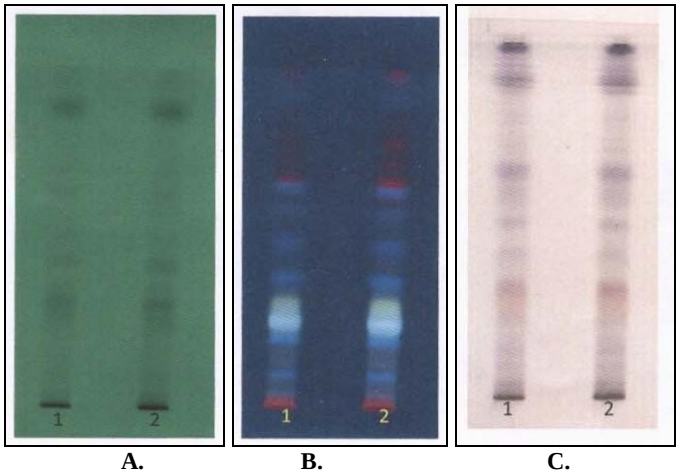
techniques. In the last few decades, HPTLC has become an important tool for the qualitative and quantitative phytochemical analysis of herbal drugs and formulations. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of marker compound and mobile phase with very less time [3]. The finger print profile of the bioactive compounds obtained in the study could be used for the quality control purpose of the formulation.

TABLE 3. HPTLC FINGER PRINT PROFILE - SCANNING AT 520 nm

Peak	Start Position	Start Height	Max. Position	Max. Height	Max %	End Position	End Height	Area	Area %
1.	0.03 Rf	0.9AU	0.04Rf	18.1AU	1.17%	0.5 Rf	0.4AU	169.2 AU	0.44%
2.	0.07 Rf	0.5AU	0.09Rf	20.0AU	1.29%	0.10 Rf	0.6 AU	250.3 AU	0.58%
3.	0.11 Rf	0.2AU	0.13Rf	32.9AU	2.13%	0.14 Rf	9.7 AU	588.5 AU	1.38%
4.	0.15 Rf	9.8AU	0.15Rf	11.0AU	0.71%	0.17 Rf	0.2 AU	142.4 AU	0.33%
5.	0.18 Rf	0.1AU	0.29Rf	144.2AU	9.31%	0.34 Rf	5.2 AU	8701.5 AU	20.15%
6.	0.34 Rf	5.4AU	0.38Rf	48.0 AU	3.10%	0.42 Rf	3.0 AU	1725.0 AU	3.99%
7.	0.42 Rf	3.1AU	0.47Rf	81.4 AU	5.25%	0.50 Rf	12.0 AU	2417.9 AU	5.60%
8.	0.50 Rf	127AU	0.54Rf	78.1 AU	4.91%	0.56 Rf	71.5 AU	2258.1 AU	5.23%
9.	0.56 Rf	70.4AU	0.60Rf	166.8AU	10.75%	0.65 Rf	0.8 AU	6572.0 AU	15.22%
10.	0.65 Rf	1.0 AU	0.66Rf	26.1 AU	1.69%	0.69 Rf	6.3 AU	461.1 AU	1.07%
11.	0.69 Rf	7.0 AU	0.69Rf	22.2 AU	1.44%	0.71 Rf	0.5 AU	202.2 AU	0.47%
12.	0.73 Rf	0.2 AU	0.75Rf	33.6 AU	2.17%	0.79 Rf	7.4 AU	762.2 AU	1.77%
13.	0.80 Rf	5.4 AU	0.82Rf	158.4AU	10.22%	0.83 Rf	49.9 AU	2406.3 AU	5.57%
14.	0.83 Rf	150.4AU	0.84Rf	295.4AU	19.06%	0.88 Rf	50.4 AU	7753.9 AU	17.96%
15.	0.88 Rf	153.5AU	0.88Rf	167.8AU	10.22%	0.90 Rf	34.9 AU	2687.2 AU	6.22%
16.	0.92 Rf	122.1AU	0.94Rf	247.5AU	15.98%	0.96 Rf	0.1 AU	6063.0 AU	14.04%

TABLE 4: HPTLC FINGER PRINT PROFILE - SCANNING AT 254nm

Peak	Start Position	Start Height	Max. Position	Max. Height	Max %	End Position	End Height	Area	Area %
1.	0.03 Rf	0.2AU	0.04Rf	33.2AU	2.88%	0.05 Rf	0.3AU	283.7 AU	0.71%
2.	0.07 Rf	4.9AU	0.09Rf	25.4AU	2.20%	0.11 Rf	0.2 AU	374.1 AU	0.90%
3.	0.15 Rf	0.1AU	0.17Rf	6.2AU	0.71%	0.19 Rf	0.2 AU	167.9 AU	0.40%
4.	0.19 Rf	0.7AU	0.25Rf	188.6AU	16.35%	0.27 Rf	36.7 AU	5734.4 AU	13.83%
5.	0.27 Rf	167.2AU	0.30Rf	218.4AU	18.94%	0.38 Rf	9.8AU	9128.3 AU	22.01%
6.	0.37 Rf	10.2AU	0.40Rf	150.3AU	13.04%	0.45 Rf	1.7 AU	4414.0 AU	10.65%
7.	0.45 Rf	1.8AU	0.48Rf	40.5AU	3.51%	0.51 Rf	0.0 AU	997.2 AU	2.40%
8.	0.52 Rf	1.9AU	0.53Rf	13.7 AU	1.19%	0.54 Rf	11.6 AU	147.9 AU	0.36%
9.	0.57 Rf	10.8AU	0.50Rf	79.2AU	6.57%	0.64 Rf	15.9AU	2608.0 AU	6.29%
10.	0.65 Rf	12.2AU	0.56Rf	21.9 AU	1.90%	0.68 Rf	6.3AU	452.2AU	1.09%
11.	0.72 Rf	8.1AU	0.74Rf	16.8 AU	1.46%	0.77 Rf	10.0 AU	599.6 AU	1.45%
12.	0.77 Rf	10.2 AU	0.84Rf	235.9AU	20.45%	0.88 Rf	12.9AU	10941.3AU	26.38%
13.	0.89 Rf	02.0AU	0.92Rf	121.0AU	10.49%	0.98 Rf	0.1 AU	5608.5AU	13.53%



A. B. C.

FIG.1: HPTLC FINGER PRINTING PROFILE

Track 1 -10µL, Track 2 -15µL.

A. Before derivatization under 254 nm, B. Before derivatization under 366 nm, C. After derivatization with vanillin sulphuric acid

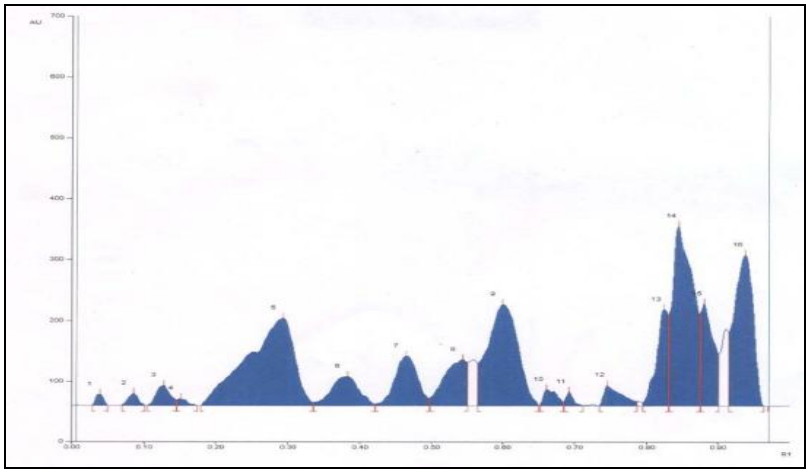


FIG. 2: HPTLC CHROMATOGRAPH OF THE ANTIDIABETIC POLYHERBAL FORMULATION - SCANNING AT 520 nm

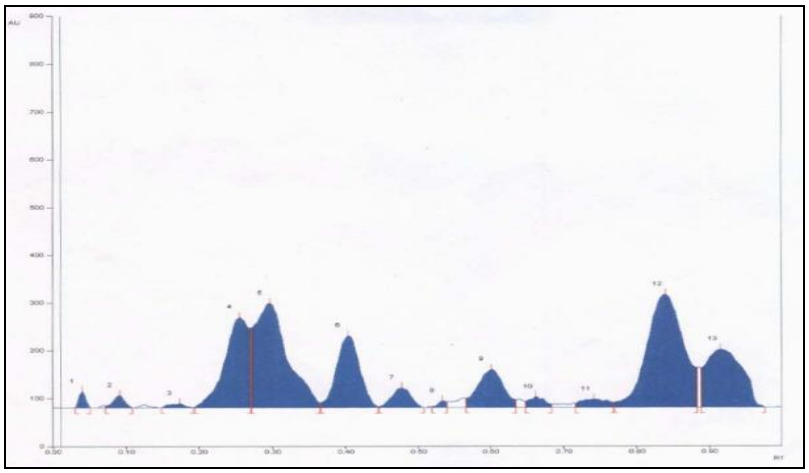


FIG. 3: HPTLC CHROMATOGRAPH OF THE ANTIDIABETIC POLYHERBAL FORMULATION- SCANNING AT 254 nm

CONCLUSIONS: The present investigation revealed the presents of bioactive compounds namely phenols, flavonoids, tannins, terpenoids, steroids, and insulin in the polyherbal antidiabetic formulation. The HPTLC fingerprint analysis confirmed that the formulation possess many phytoconstituents. The polyherbal formulation is considered to be a potential source of natural antioxidants and may be a promising candidate for the treatment of diabetes and other metabolic disorders. Further studies are needed to investigate the pharmacological profile of the formulation using in vitro and in vivo models.

CONFLICT OF INTEREST: The authors declared that they have no conflict of interest.

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