Quantitative Determination of Berberine in Marketed Herbal Formulations by TLC Densitometry and their Hypoglycemic Effect

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ABSTRACT

In the present time, a phenomenal increase in the demand for herbal medicine has been observed and a need has been felt for insuring the quality, safety and efficacy of herbal drug preparations. Standardisation is one of the tools for the assessment of quality, purity and efficacy which includes preliminary phytochemical screening, fingerprint profile using biomarkers corroborated with modern analytical techniques. HPTLC has established itself as an important tool for handling the qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations due to its simplicity, flexibility, reliability and cost efficient separation. Four ayurvedic formulations Nimbadi Churna (Vyas Pharmaceuticals), Pushyanug Churna (Baidyanath Ayurved Bhavan), Khadirarishta (Sandu Pharmaceuticals Ltd.), Trifla Ghrita (AVS Kottakkal) have been selected in the present study. The use of these formulations varies from blood purification, bleeding disorders to eye related diseases. These also act as anti-inflammatory, antibacterial and anthelmintic agents. Berberine is one of the major constituents in all the selected formulations. The major therapeutic activity of berberine is for the treatment of infection and inflammation, however, the antidiabetic potential of berberine is also under scrutiny. In the present study, quantitative determination of berberine was carried out in all the formulations by TLC densitometric analysis. Further, the anti-diabetic activity of these formulations was evaluated by streptozotocin (STZ) induced rats using glimepiride and berberine as a standard drug. All the formulations at a dose level of 45 mg/kg showed significant hypoglycemic activity and formulation-III, showed better activity as compared to the standard drug. Hence these formulations can be used in the treatment of diabetes beyond their usual activities.

Keywords: Nimbadi Churna, Pushyanug Churna, Khadirarishta, Trifla Ghrita, Berberine, TLC densitometry, hypoglycemic activity.

INTRODUCTION

One of the major impediments in the acceptance of the Ayurvedic or herbal medicines is the lack of standard quality control profiles[1-3]. It is difficult to establish quality control parameters of the plant based drugs due to the complex nature and inherent variability of the chemical constituents[4,5]. There are several problems which influence the quality of herbal drugs.[6]

Variable sources of the raw material.

The chemical constituents of herbs and herbal products may vary depending on stage of collection, parts of the plant collected, harvest seasons, drying processes and other factors.

Extracts are usually mixtures of many constituents[7,8].

Standardization of plant materials is the need of the day. Several Pharmacopoeia containing monographs of the plant materials describe only the physicochemical parameters. The modern methods describing the identification and quantification of active constituents in the plant material may be useful for proper standardization of herbal formulations[9-10]. The international organizations like WHO has emphasized the need to ensure the quality of medicinal plant products using modern analytical techniques compared with suitable standards[9]. HPTLC offers quantitative estimation of active constituents with better accuracy, precision and resolution in a shorter time[13-17]. This technique can be quite helpful in setting up of standards for evaluating the purity and quality of Ayurvedic preparations and to overcome batch to batch variations in these formulations[18].

Berberis aristata commonly known as "Daruhaldhi and Chitra" is spinous herb native to northern Himalaya region. It is also known as Indian Barberry or Tree Turmeric, belonging to the family Berberidaceae and the genus *Berberis*. *B. aristata* is used in ayurvedic medicines from very long time for the treatment of infection and inflammation. The plant is used traditionally in inflammation, wound healing, skin disease, menorrhagia, diarrhea, jaundice and infection of eyes[19]. *B. aristata* contains protoberberine and bis-isoquinoline type of alkaloid. Root of plant *B. aristata* contains alkaloid which are berbamine, berberine, oxycanthine, epiberberine, palmatine, dehydrocaroline, jatrorhizine and columbamine, karachine. The major alkaloid found in *B. aristata* is berberine having yield of 2.23% followed by palamatine[20].

The anti-diabetic effect of berberine was noted in 1988 during the treatment of gastro-intestinal infections in patients with diabetes[21]. Till then many researchers have explored the anti-diabetic activity of berberine in streptozotocin induced diabetic rats[22,23]. Beberine is the major constituent of many herbal formulations existing in ayurvedic system. The common formulations containing berberine are Khadirarishta, Trifla Ghrit, Pushyanug Churna, Nimbadi Churna. Nambai churna is an excellent blood purifier, best for all types of skin diseases, dandruff, joints pains and pyorrhoea[24]. Pushyanug churna is used in the ayurvedic treatment of bleeding disorders. It is anti-inflammatory (reduces swelling) and anti-spasmodic (relieves spasm) in the uterus. It is a circulatory and menstrual regulator[25]. Khadirarishta has blood purifying, laxative, anthelmintic antibacterial, digestive and pitta pacifying action and therefore eliminates blood toxins[26]. Trifla Ghrita is useful in excessive discharge in women, eye pain with itching and discharge, pterygium (conjuctiva) and other eye and eye lid related diseases. It is also used in cough, oedema, hair fall and intermittent fever[27].

Diabetes affects the people from all age groups, so it was thought that diabetic patients with inflammation, wound healing, skin disease, menorrhagia, diarrhea, jaundice and infection of eyes can be treated with these formulations with better efficacy and potency. Thus this study was undertaken to explore the efficacy of anti-diabetic activity of the selected ayurvedic formulations (Nimbadi Churna (Vyas Pharmaceuticals), Pushyanug Churna (Baidyanath Ayurved Bhavan), Khadirarishta (Sandu Pharmaceuticals Ltd.), Trifla Ghrita (AVS Kottakkal)) in diabetic rats and quantitative estimation of berberine in the selected ayurvedic formulations.

MATERIALS AND METHODS
Materials
Berberine was obtained from Alfa Aesar, Thermo Fisher Scientific, India. All other chemicals and solvents (Merck Specialities Pvt. Ltd., S.D. Fine Chemicals and Himedia Laboratories Pvt Ltd, India) were of analytical grade and procured locally. Marketed formulation Nimbadi Churna (Vyas Pharmaceuticals): Formulation-I, Pushyanug Churna (Baidyanath Ayurved Bhavan): Formulation-II, Khadirarishta (Sandu Pharmaceuticals Ltd.): Formulation-III, Trifla Ghrita (AVS Kottakkal): Formulation-IV were purchased from local market. Glimepiride was obtained as gift sample from Consern Pharma Pvt. Ltd., Ludhiana, Punjab.

Preliminary phytochemical screening
The formulations (5 g) were extracted by maceration with 100 mL of methanol at room temperature. The extract was filtered, evaporated to dryness and subjected to preliminary phytochemical screening for alkaloids, steroids, carbohydrates, tannins, flavonoid glycosides as per standard pharmacognostic methods[28-30].

TLC fingerprint profile
The comparative TLC chromatograms and fingerprint profile of different formulation and standard berberine were developed using pre-coated silica gel F_{254} plates [E.

Merck (India) Ltd., alumina base, 0.2 mm thickness] and using the solvent systems saturated with the mobile phase n- propanol: formic acid: water (16.2: 0.2: 3.6). The developed TLC plates were heated at 110 °C for 5 min or till the bands developed colour. The TLC fingerprint profiles were recorded as images under UV at 254, 366 nm and under white light. The TLC fingerprint chromatograms were recorded for comparison and an overlay of the densitometric scan of different formulation with berberine was obtained.

TLC densitometry study
The samples were applied as bands and keeping a distance of 6 mm between the bands, on precoated silica gel G aluminium plate 60F254 (20 cm × 10 cm, 0.2 mm thickness; Cat. no. 1.05554.0007, E. Merck, Darmstadt Germany, Ltd.) using Linomat 5 (Camag, Switzerland). The plates were prewashed by methanol and activated at 60 °C for 5 min prior to chromatography. The mobile phase consisted of n-propanol: formic acid: water (16.2: 0.2: 3.6) and the plate was developed ascendingly in twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with mobile phase. The chamber saturation time was 10 min with room temperature (25 °C ± 2) and a relative humidity of 60% ± 5. The development distance was kept at 8 cm. The developed TLC plate was air dried and scanning was performed on Camag TLC scanner 3 in the reflectance absorbance mode at 350 nm by WINCATS software (Camag, version 4.06). Berberine concentration was determined in all the formulations from the intensity of diffusely reflected light and evaluation was done via peak areas with linear regression.

Calibration curve of berberine
The standard solution of berberine (10 mg/10 ml) was prepared in methanol in a 10 ml volumetric flask and further serially diluted to get different concentration of 10-50 ng/μL. Each concentration was spotted three times on the TLC plate. 1 μL of the standard solution was applied on the TLC plates.

Quantification of berberine in marketed formulations
The test samples (1μl) were applied and chromatogram was obtained under same conditions as that of standard berberine. The area of peak corresponding to the R_f value of standard berberine was recorded and the amount present was calculated from regression equation obtained from calibration curve.

Pharmacological activity
Experimental animals
The animal studies were approved by the Institutional Animal Ethics Committee (IAEC) and were used according to the CPCSEA (Committee for the Purpose of Control and Supervision of Experimentation on Animals) guidelines. Wistar/SD rats weighing ≈200-250 g were used in the study. The animals were kept in animal cages, 6 rats per cage, on straw bedding in an animal house maintained natural controlled environment of temperature (30 ± 2 °C) and humidity (50 ± 10%). The animals were fed on standard rodent food pellets diet and water ad libitum. After one week of acclimatization, animals were randomly distributed into different experimental groups. The test and standard doses were administered orally with the help of

Table 1: Preliminary phytochemical screening of different extracts.

S. No.	Constituents	Test performed	Result
1.	Alkaloids	Dragondorff's test	+ve
2.	Carbohydrates	Molish test	+ve
3.	Glycosides	Borntrager's test	+ve
4.	Flavonoids	Shinoda test	-ve
5.	Saponin	Froth formation	-ve
6.	Tannins	Gold beater's test	+ve
7.	Amino acids	Millon's test	-ve
8.	Proteins	Biuret test, warming test	+ ve

Table 2: Content of berberine in the formulations.

S. No.	Sample	Amount of Sample Application	AUC $\pm$ SD	Value obtained in ng	Value obtained in mg
1.	Nimbadi Churna	1µl	6577.6±305.5	119.01	0.119
2.	Pushyanug Churna	1µl	9095169.6±258	159702.38	159.7
3.	Khadirarishta	1µl	172428.5±2338.5	3031.13	3.031
4.	Trifla Ghrita	1µl	3755.5.1±317.27	69.47	0.069

The results are expressed as mean (n=3) $\pm$ Standard deviation (SD).

Table 3: Comparison of content of berberine obtained and reported in different formulations.

S. No.	Sample	Berberine claimed in the formulations (mg)	Berberine content (mg)
1.	Nimbadi Churna	0.139	0.119
2.	Pushyanug Churna	192.3	159.7
3.	Khadirarishta	4.50	3.031
4.	Trifla Ghrit	0.078	0.069

Table 4: Hypoglycemic activity of different extracts of ayurvedic formulations

Group	Treatment	Blood Glucose Level (mg/dl)		% inhibition
		0 day	19th day	
I	Diabetic Control	650±1.04	331.3±6.98	-
II	Glimepiride (Std)	630.51±7.45	130.57±0.72	60.59
III	Berberine	546.36±14.20	148.57±1.61	55.15
IV	Formulation-I	585.28±20.35	194.63±4.95	41.25
V	Formulation-II	457.82±35.89	219.67±3.18	33.69
VI	Formulation-III	668.71±12.28	181.27±4.72	45.28
VII	Formulation-IV	602.93±4.49	280.58±5.30	15.31

The results are expressed as mean (n=3) $\pm$ Standard deviation (SD).

an oral cannula fitted on a tuberculine syringe and streptozotocin was injected intra peritonially.

Experimental design and induction of diabetes

Diabetes was induced by injecting 45 mg/kg body weight of streptozotocin (STZ) dissolved in citrate buffer solution of pH 7.4 intra-peritoneal. The animals are kept under observation and after 72 h blood sugar was be measured by glucose kit (Erba diagnostics Mannheim Germany). The rats having blood glucose, higher than 200 mg/dl, were considered as diabetic for experiment. The diabetic rats (glucose level 200-300 mg/dl) were separated and divided into 7 groups containing 6 animals in each group. Group I (glucose control); group II and III (standard control i.e. berberine, glimepride); group IV, V, VI and VII (extracts of different marketed formulations at the dose of 45 mg/kg). After 21 days of consecutive dosing of different formulation and standard drug, the blood was collected from retro-orbital vein of eye of each rat in anesthetic condition. Blood glucose was measured using a glucose kit (Erba diagnostics Mannheim Germany).

Statistical analysis

All the grouped data were statistically evaluated with hypothesis testing methods including one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as mean $\pm$ SD. The statistical analysis was done using the Graph Pad Prism statistical software version 5.

RESULTS

Phytochemical analysis

The methanolic extracts of all the marketed formulations showed the presence of alkaloids, glycosides, tannins, carbohydrates and proteins (Table 1).

Calibration curve of standard berberine

The HPTLC procedure was optimized by varying the ratio of solvents in the solvent system consisting of n-propanol, formic acid and water. Finally, a solvent system containing n-propanol: formic acid: water in the ratio 16.2: 0.2: 3.6

was selected which gave a sharp and well defined peak at R_f = 0.64. Different concentrations (10 ng/µl - 50 ng/µl) of

standard solution of berberine (standard marker compound) were applied on HPTLC plates and calibration
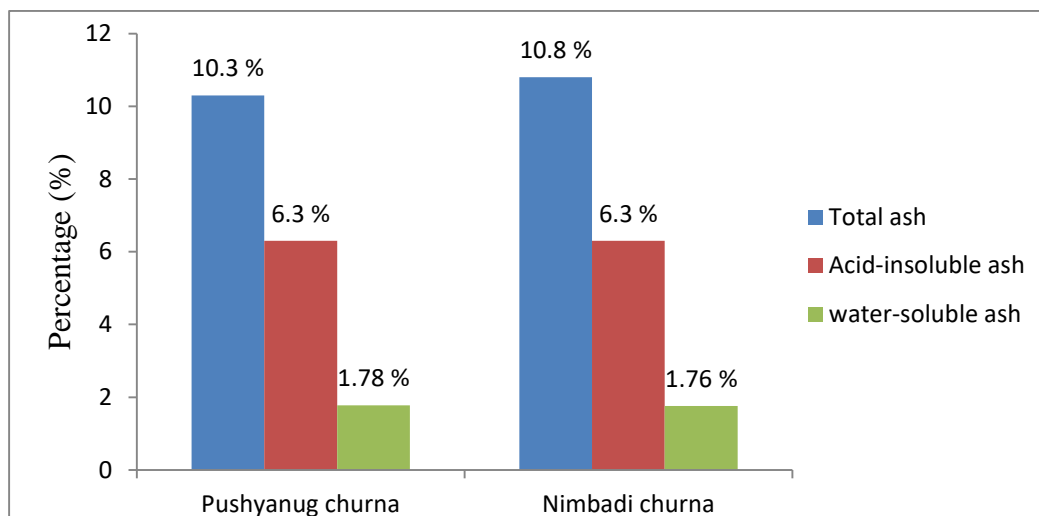

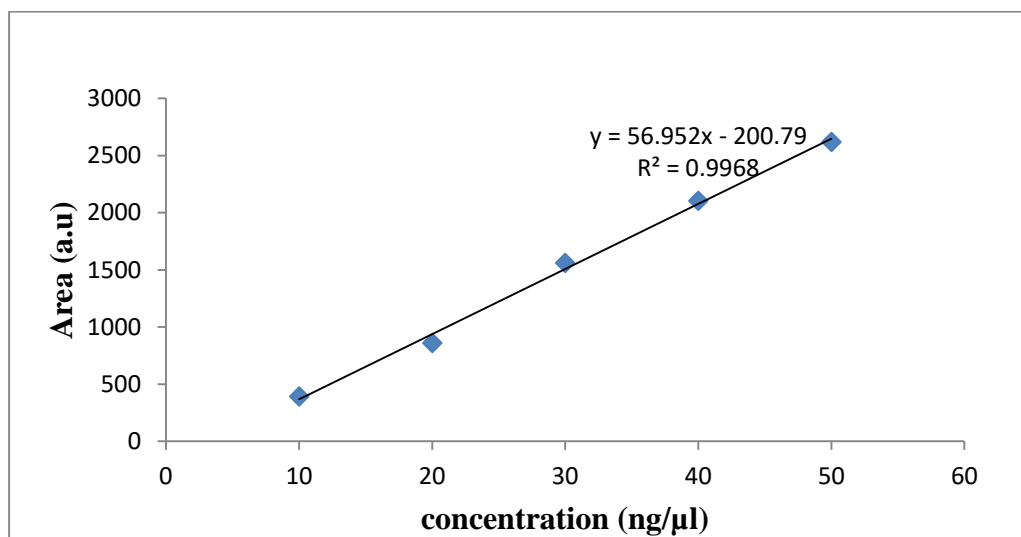Figure 1: Graphical representation of ash values for different marketed formulations.
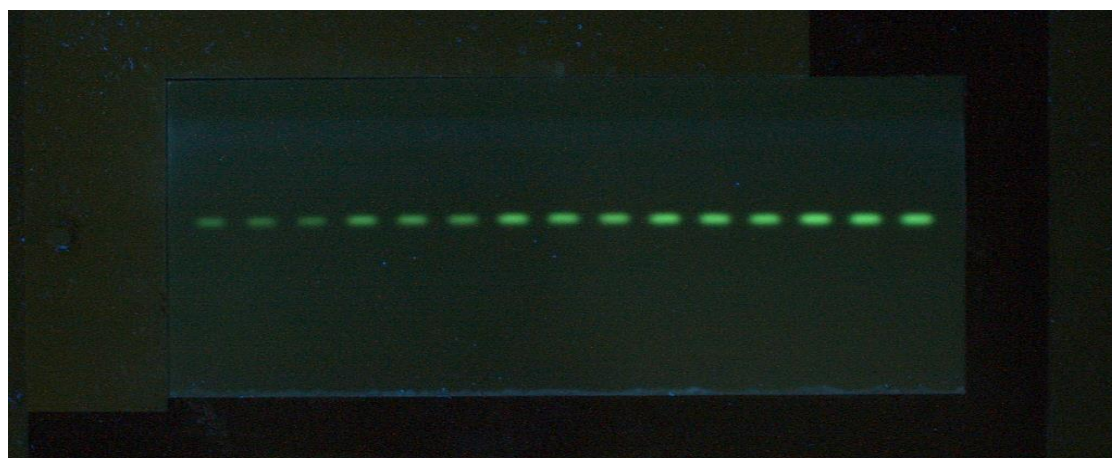

Figure 2: Standard calibration curve for berberine.

Figure 3: HPTLC plate of standard berberine seen at 323 nm in UV light.
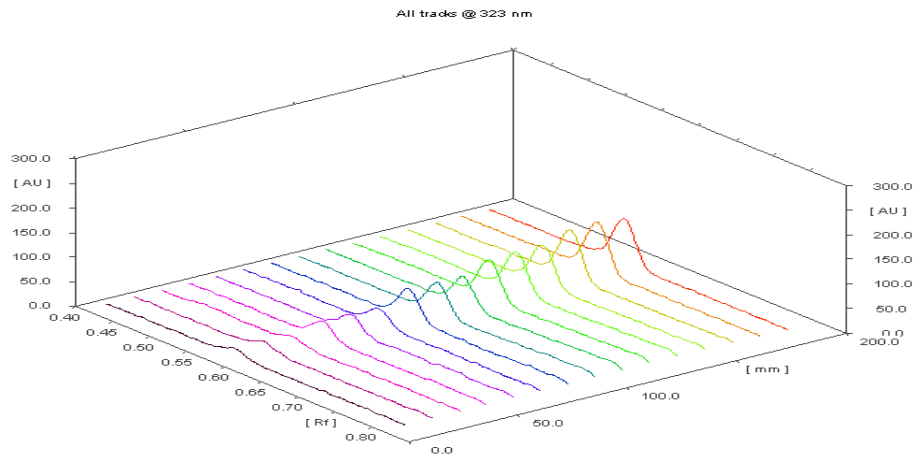
Figure 4: Three dimensional image of the calibration spots for berberine at 323 nm.
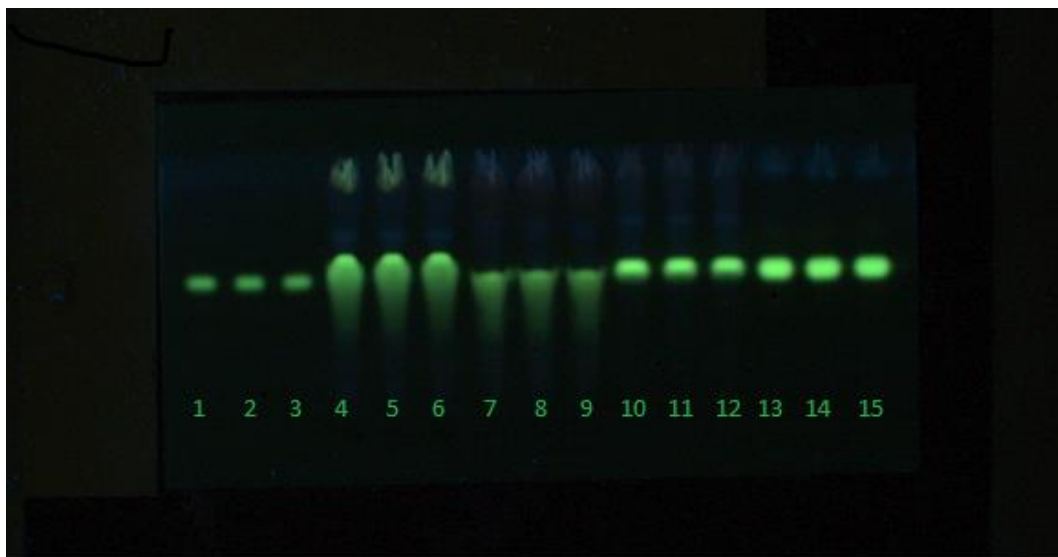

Figure 5: TLC densitometric profile of marketed formulations. Visualisation: UV 323 nm. Tracks: (1,2,3) std. berberine, (4,5,6) Nimbadi Churna, (7,8,9) Pushyanug Churna, (10,11,12) Khadirarishta, (13,14,15) Trifla Ghrit.
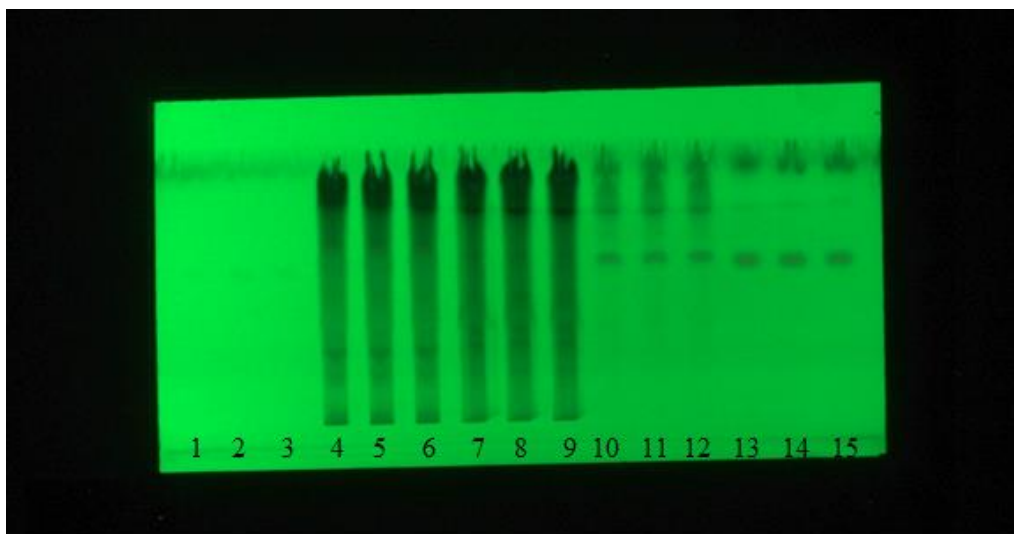

Figure 6: TLC densitometric profile of marketed formulations. Visualisation: white light Tracks: (1,2,3) std. berberine, (4,5,6) Nimbadi Churna, (7,8,9) Pushyanug Churna, (10,11,12) Khadirarishta, (13,14,15) Trifla Ghrit.

curve was prepared by plotting peak area against amount of berberine applied. In the present analysis calibration curve of standard berberine was found to be linear and the linear regression equation obtained was y = 56.952x−200.79 with a correlation coefficient (r) of 0.9968 (Figure 1). HPTLC photograph of standard berberine and formulations is presented in figure 2 and 3.

Estimation of berberine in marketed formulations

Berberine peaks in the methanolic extracts of all the marketed formulations were identified by comparing their single spot at R_f = 0.64 values with those obtained by chromatography under the same condition. The overlay spectra of identified peaks in the standard as well as the corresponding peaks in the extracts of marketed formulations showed total super imposition. Quantitative analysis of berberine in the formulations was performed through HPTLC techniques and result showed that content of berberine in the formulations was found to be 85.6, 83.0, 67.3 and 88.4 % compared to standard berberine. The respective HPTLC chromatograms of berberine and all the formulations are presented in the figure 4 and 5. The total berberine content was found to be 0.119, 159.7, 3.03, 0.069 mg in formulations I, II, III and IV respectively. However, the content claimed in the formulations is 0.139, 192.3, 4.5, 0.078 mg respectively for formulations I, II, III and IV. The content of berberine present in polyherbal formulation is shown in table 2. From table 3 it is clear that there is a wide variation in the content of berberine in all the formulations.

Anti-diabetic activity

The anti-diabetic activity of the Ayurvedic formulations (I, II, III and IV) was evaluated by measuring blood glucose level in streptozotocin induced diabetic rats using glimepiride and berberine as standard drugs. The results were expressed as mean±SD difference between vehicle control and treatment groups were tested using one way ANOVA followed by the least significant difference (L.S.D). All the four extracts (formulation I-IV) showed significant hypoglycemic activity in STZ induced diabetic rats at dose of 45 mg/kg body weight, but the formulation III (181.27±4.72) showed the best activity among all the formulations in lowering of blood glucose level as compared with the standard drug berberine (546.36±14.20) and glimepiride (630.51±7.45). The results are shown in table 4.

DISCUSSION

Type 2 diabetes mellitus is a complicated metabolic disorder characterized by impairment of both glucose utilization and gluconeogenesis. It has been reported that berberine has activity comparable to sulphonureas and metformin in reducing blood glucose in diabetic patients in China. In Indian system of medicine formulations containing berberine have been used for a number of ailments. The four berberine containing ayurvedic formulations used in the present study showed significant anti-diabetic activity in STZ induced hypoglycemic rats with upto 45 % decrease in blood glucose levels. STZ causes diabetes by the rapid depletion of β-cells and thereby brings about a reduction in insulin release. All these polyherbal formulations can be utilized in diabetic patients with other therapeutic manifestations such as infection, inflammation and other skin and menstrual disorders.

Herbal medicines are complex chemical mixtures obtained from a plant which are widely used in health-care in both developed and developing countries. Herbal medicines contain more than one principle compound and it is important that the constituent responsible for activity should be quantified and standardized using a reference drug. Standardization is the process of delivering a product with a specified level of one or more phyto-constituents which broadly covers the qualitative and quantitative analysis. HPTLC is a modern adaptation of TLC with better and advanced separation efficiency and detection limits. It offers better resolution and estimation of active constituents can be done with reasonable accuracy in a shorter time. In the present study, TLC densitometric analysis of the four formulations was performed. HPTLC study confirmed the qualitative as well as quantitative presence of the raw material in the finished product. However, the results revealed that the content of berberine in the formluations was different from what has been claimed. This shows that these polyherbal formulations are not standardized which can lead to difference in therapeutic efficacy of these formulations. Thus these formulations must be standardized using quantitative analytical method as observed in the present study.

CONCLUSION

Berberine, used for treatment of infection and inflammation has a promising antidiabetic activity. The four formulations containing berberine showed significant hypoglycemic activity in STZ induced diabetic rats. This suggests that these formulations can be used effectively in diabetes control. Also the standardization of the polyherbal formulations containing berberine by TLC densitometry has shown that contents may vary from the claimed contents. Thus an official procedure must be generated to estimate the contents of polyherbal formulations to make the Indian traditional system of medicine the treatment of choice for many diseases.

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