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HPTLC quantification of triterpenoid compounds and evaluation of *in-vitro* potential of *Trichodesma indicum* L

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Abstract
Trichodesma indicum (Linn.) R.Br. is traditionally used folk medicine, having a lot of pharmacological bustles. The current study is focused on investigating the phytochemical and biological evaluation of *T. indicum*. Quantification of oleanolic acid, lupeol, and linolic acid is done by using a tertiary mobile phase consisting of toluene: ethyl acetate: formic acid (7: 2.5: 0.5 v/v). Oleanolic acid, lupeol, and linolic acid were resolved at R_f 0.46 ± 0.04, 0.55 ± 0.04 and 0.62 ± 0.05 respectively and it was quantified at its wavelength of maximum absorbance of 600nm after derivatization with anisaldehyde sulphuric acid reagent. The limits of detection and limit of quantification were found to be 662.4, 665.18 and 660.26ng per ml and 2007.4, 2015.7 and 2000.78 ng per ml for linolic acid lupeol, and oleanolic acid, respectively. The linear regression data for the calibration plot showed a good linear relationship between peak area and concentration in the range of 200-600ng/Spot. The developed HPTLC method is accurate, precise and has been successfully applied for the assay of this bioactive molecule in *Trichodesma indicum.* The pharmacological potential of the extract is dependent on the contents of active secondary metabolites present in the plants. The antioxidant potential was studied by using DPPH and 2-deoxyribose assay. Antioxidant potential was observed showing IC_{50} values (326.82±1.21µg/ml) and (449.62±2.01µg/ml) by DPPH and 2-deoxyribose method respectively. *In vitro* antidiabetic potential of the plant were also assessed by starch iodine assay and DNS method. IC_{50} was observed (86.57 ± 2.57µg/ml) in 3, 5 DNS assay and (222.86 ± 3.36 mg/ml) in starch iodine assay. Results are promising; this work forms a firm base for further research to explore the lead compound which is responsible for the medicinal value of this plant.

Keywords: *Trichodesma indicum,* DPPH, antidiabetic, linolic acid, lupeol etc

Introduction
Medicinal plants have always been part of human society and have the potential to cure many ailments caused by various factors. Since ancient times, many plants have been used by people for the treatment of various diseases and disorders. Tribal medicine plays an important role in the primary health care of tribal as well as rural people (Khalil S A., 1995) [20]. Therefore, it is essential to document the unique properties of plants as well as tribal knowledge to give a scientific understanding that how these plants can cure certain ailments.
Trichodesma indicum (Linn.) R.Br. (Boraginaceae) is commonly known as "Adhapushpi", in Gujarati known as "Undhaphuli" found as a weed throughout the greater part of India and distributed in tropical and subtropical part of Asia, Africa and Australia (Verma N V *et al.*, 2010) [1]. *T. indicum* is used in arthritis, dysentery, skin diseases, snake-bite poisoning, fever, cough reflux depressant, blood pressure, rheumatism and in weakness (Parrotta A.J. *et al.*, 2001; Varier S.P.V *et al.*, 1993, Ghisalberti E.L. 2005, Srikanth, K. *et al.*, 2002) [3, 4, 5, 6]. In Ayurveda, *T. indicum* is beneficial in eye diseases, the expulsion of the dead fetus and reduce swelling especially in joints (Saboo S.S. *et al.*, 2012) [7]. It is also used as antimicrobial, anti-inflammatory, immune stimulating, antineoplastic, antiviral and antidiarrheal, (Perianayagam JB *et al.*, 2012, Perianayagam JB *et al.*, 2005) [11, 12]. Phytochemically, it contains monocrotolin, suspinine as pyrrazolidine alkaloids, hexacosane, amylin, ß-sitosterol and catechin and gallic acid (Saboo S S *et al.*, 2013; Kirtikar, K. R. *et al.*, 1935; Hasan M. *et al.*, 1982) [9, 2, 8]. Seed oil yield oleic, palmitic, stearic, and linolenic acid (Asad *et al.*, 2013) [10].
As per the report *T. indicum* is acrid and bitter in taste. It is an erect, spreading, branched and annual herb, about 55cm in height with hairs springing from tubercles. The leaves are stalkless opposite, lance shaped, 2.4-8cm long, pointed at the tip and heart shaped at the tip. The flowers occur singly in the axils of the leaves and generally violet, light blue or purple in color. The calyx is green, hairy and 1-1.3 cm long with pointed sepals. The corolla is pale blue with limb about 1.5cm in diameter and the petals are pointed.

The fruit is ellipsoid and is enclosed by the calyx. The nutlets are about 5mm long and rough on the inner surface (Hamsalakshmi *et al.*, 2018).
Heans, *Trichodesma indicum* R. Br has been explored for microscopical, physic-chemical, phytochemical and varied pharmacological investigations; however, there are no reports published for the identification of triterpenoid and *in-vitro* antidiabetic analysis. The current study is designed for quantification of linolic acid, oleanolic acid and lupeol through HPTLC and evaluation of the pharmacological potential of *T. indicum* using various *in-vitro* assays. This will serve as reference material for any further studies related to identification, adulteration, pharmacognostical and phytochemical investigations.

Material and Methods

Plant material

Trichodesma indicum collected in the month of September, 2015 from Sirmour, Himanchal Pradesh. Sample was authenticated by Dr. Manoj tripathi, (HOD) Deendayal research Institute, Chitrakoot and voucher specimens (Voucher no AD/AS/104/2015) were deposited in institute's herbarium. Collected samples were washed, shade dried and powdered for further studies.

Standard solutions and reagents

Ascorbic acid (99%), gallic acid (99%), (MP Biomedicals India Pvt Ltd. Navi Mumbai, Maharashtra) toluene, methanol, ethyl acetate, formic acid (Spectrochem Pvt. Ltd. Mumbai) lupeol (94%), linolic acid (99%), 1-1-diphenyl-2-pic-rylhydrazyl (DPPH), α-amylase, 3, 5-Dinitrosalicylic acid (98%), starch soluble, iodine (99.99%), aluminium chloride (99.99%), sodium carbonate (99%), Folin's reagent (97%) were purchased from (Sigma-Aldrich, India). All other solvents and chemicals (AR grade) are obtained from SD Fine Chemicals, Mumbai, India.

Preparation of plant extracts

The plant material was manually screened for any impurities and dried in shade. Sample was powered with an electric grinder and coarse powder was subjected to methanolic extraction. Extracts were continuously stirred for 6 hrs and kept up to 18 hrs at room temperature. The process was repeated up to complete extraction. The extract was filtered and concentrated under vacuum in a rotatory evaporator (Buchi rotavpour, Switzerland) at 45 °C. The extract was finally freeze dried and stored at 4 °C for further use.

In vitro activities

In-vitro antioxidant activity

Total flavonoid and phenolic content was estimated (Ordon Ez, 2006) [13] and expressed in terms of mg/g of QE (Quercetin Equivalent) and mg/g GAE (Gallic Acid Equivalent) based on calibration curve of Quercetin and Gallic acid as standard. The anti oxidant potential was analyzed via DPPH radical scavenging assay (Shukla *et al.*, 2016) [15] and 2 deoxy ribose (S Ganapaty *et al.*, 2013) [18].

Alpha amylase inhibition assay

Starch-iodine assay

Assay was carried out with slight modification based on the starch-iodine test (Xiao *et al.*, 2006) [17]. Inhibition of enzyme activity was calculated as:

Inhibition of enzyme activity (%) = (C-S) / C × 100

where S is the absorbance of the sample and C is the absorbance of blank (no extract).

3, 5-Dinitrosalicylic acid method (DNS)

The inhibition assay was performed using DNS method (Miller, 1959) [16]. The results were expressed as % inhibition calculated using the formula.

Inhibition activity (%) = Abs (Control)-Abs (extract) × 100 / Abs (Control)

High Performance Thin Layer Chromatography

Preparation of standard compound solution

The stock solution of lupeol, linolic acid and oleanolic acid (1 mg/ml) was freshly prepared in analytical grade methanol. A stock solution of 1000 µg/mL of lupeol, linolic acid and oleanolic acid were diluted with same solvent to obtain a four working solutions of concentration ranging from 200µg/ml, 400µg/ml and 600µg/ml for further analysis. The solutions were filtered through a 0.45 µm Millipore membrane filter (Pall, USA) before application. 10 mg/ml of the plant extract was used for HPTLC studies.

HPTLC conditions

High performance thin layer chromatography is used for separation of the components present in alcoholic extract of the plant, both quantitatively as well qualitatively. For quantitative analysis 10mg/ml, about 10µl sample was applied using Camag 100 ml sample syringe (Hamilton, Switzerland) on pre-coated plates with silica gel 60F $_{254}$ of 0.2 mm thickness as 6 mm-wide bands positioned 10 mm from the bottom and 15 mm from side of the plate, using CAMAG LINOMAT V automated TLC applicator with nitrogen flow providing a delivery speed of 150nl/s from application syringe. Plate was developed in a CAMAG twin trough glass chamber which was pre-saturated with mobile phase Toluene: Ethyl acetate: Formic acid (7: 2.5: 0.5 v/v). After development of the plate, it was dried and then derivatized with anisaldehyde-sulphuric acid and scanned at 600nm with a TLC scanner (WINCATS 1.3.2, CAMAG).

Calibration

An amount of 2 ml of three calibration standards was achieved with a concentration range of 200, 400, 600 ng/spot of each standard compounds lupeol, linolic acid and oleanolic acid. TLC plates developed to the solvent system to a distance of 85mm. Dried the plate to obtained the chromatogram and determined the area of peak corresponding to that of lupeol, linolic acid and oleanolic acid as described given bellow for the calibration curve by plotting peak area Vs concentration of lupeol, linolic acid and oleanolic acid. This operation was repeated on three different days in order to select the most appropriate regression model for the response function.

Statistical Analysis

Results were expressed as mean ± S.D. Linear regressions analysis was carried out for standards to calculate total phenolic and flavonoid content. IC_{50} values were obtained by graph pad prism 5 software.

Result and Discussion

Medicinal plants are one of the foremost sources of dietary supplements that assist in maintaining good health (Nasri H *et* *al*., 2004). Previous phytochemical analysis reveals that bioactive compounds are mainly responsible for the potential of the plants (Altemimi A *et al*., 2017) [22]. Secondary metabolites of *Tricodesma indicum* were well recognized for their beneficial health effect. In the present investigation, quantification of bioactive compounds and evaluation of biological potential in the aerial part of the plant was made.

Analysis of phenolic and flavonoid were performed and found 11.025 mg/gm and 1.012 mg/gm respectively in *T. indicum* (Table 1). Quantification of linolic acid, lupeol and oleanolic acid were done by HPTLC in *T. indicum* on the basis of a calibration curve of standards. Three dilutions of standard were used in a concentration range of 200-600 ng/Spot and various calibration values were obtained. Maximum concentration of linolic, lupeol and oleanolic acid were found (0.0132 ± 0.002 µg/ml) (0.0352 ± 0.005 µg/ml) and (0.0042±0.031) respectively. Separation was done by using a tertiary mobile phase consisting of toluene: ethyl acetate: formic acid (7: 2.5: 0.5 v/v). Oleanolic acid, lupeol, and linolic acid were resolved at R_f 0.46 ± 0.04, 0.55 ± 0.04 and 0.62 ± 0.05 respectively and it was quantified at wave length 600nm after derivatization with anisaldehyde sulphuric acid reagent. Quantitative analysis reveals that the aerial part of *T. indicum* contains secondary metabolites (lupeol, and linolic acid) in higher amount when compared with oleanolic acid (Table 2). The developed HPTLC method is accurate, precise and may be applied for the routine analysis of bioactive molecule in medicinal plant.

Since ancient times, plants have been used as remedies to treat humans as well as animal's diseases. Identified marker compounds such as linolic acid, oleanolic acid, and lupeol, are widely found in edible fruits, vegetables and medicinal plants. They have potential to induce diabetic disorder and enhance the potential of the patients against the disease (Siddique H R *et al*., 2011; Altemimi A *et al*., 2017) [23, 22]. *In-vitro* antioxidant and antidiabetic potential analysis were performed in *T. indicum* and calculated by four different models viz. total phenolic and flavonoid, DPPH method, 2-dioxy ribose having variable mechanism of action. Polyphenolic content viz. Total phenolic and flavonoid content were estimated (Table 1), TPC and TFC were calculated as (11.026±0.479mg/g GAE), (4.4±0.005 mg/g GAE) respectively. The results obtained (Table 3), indicate that *T.indicum* extract has potent antioxidant activity IC_{50} value (326.82±1.21) were achieved by scavenging abilities observed against DPPH method and IC_{50} value achieved 449.62±2.01 in 2-dioxy ribose analysis. This is found similar to standards in i.e. ascorbic acid (0.998), quercetin (0.997) and rutin (0.998). *T. indicum* showed moderate antioxidant activities with an IC_{50} which were compared with the values of standards (Gallic acid, ascorbic acid and quercetin) used. *In vitro* antidiabetic potential of the plant was assay by starch iodine color assay and 3, 5 DNS methods of alpha amylase inhibition model. Data of starch-Iodine, reveals that activity increases linearly with concentration i.e. 0.1- 0.5 mg/ml of tested plant extract. IC_{50} of *T. indicum* (86.57 ± 2.37/ml) in 3, 5 *DNS assay* and (222.86 ± 3.36 µg/ml) in *Iodine starch assay* (Figure 3), whilst acarbose exhibit IC50 at < 25 µg/ml. From the above study, it was observed that the methanolic extract of *T. indicum* have potential against diabetic, when compared to the standard drug.

Therefore, it can be concluded that *T. indicum* have potent diabetic potential and may be used as alternative herbal medicine in future.

Fig 1: A flowering twig of *Tricodesma indica*

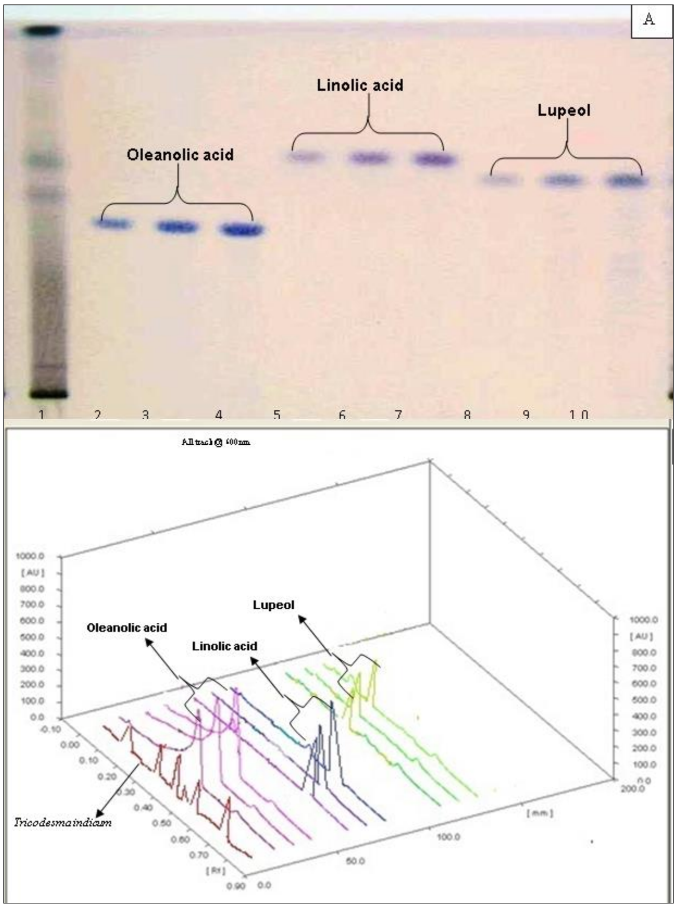

Fig 2: HPTLC finger print (A) and 3D densitometric chromatograph (B) of *Trchicodesma indicum* and standards

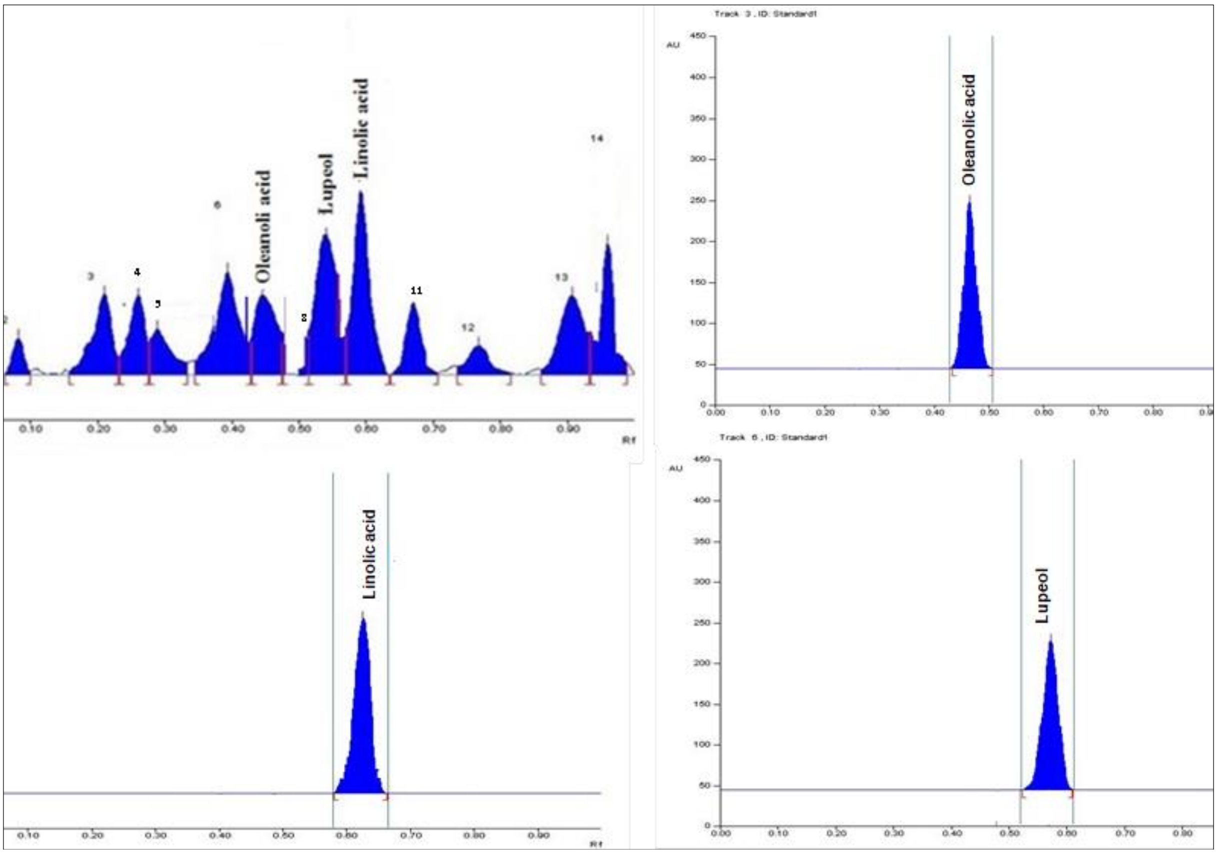

Fig 3: HPTLC chromatograph of *Tricodesma indicum*

Table 1: Total phenolic and flavonoid content

Plant	Total phenolic content (mg/g)*GAE	Total Flavonoid content (mg/g)*QE
	y = 186.34x - 0.0119 R^2 = 0.9906	**y = 81.37x - 0.080 R^2 = 0.981**
T.indicum	11.025±0.47	1.012±0.0057

(*mean ± S.D, n=3)

Table 2: HPTLC quantification of marker compound in *Tricodesma indicum*

Marker Compound	*T. indicum* Value (per dry weight) *
Linolic acid	0.0132±0.002
Lupeol	0.0352±0.005
Oleanolic acid	0.0041±0.031

(*mean ± S.D, n=3)

Table 3: IC_{50} value for *in-vitro* assay

S No.	*In vitro* assay	IC_{50} (µg/ml) *
1.	3, 5 DNS	86.57±2.378
2.	Starch- Iodine assay	222.44±3.3
3.	DPPH Assay	326.82±1.21
4.	2-dioxy ribose	449.62±2.01

(*mean ± S.D, n=3)

Table 4: Calibration parameters of marker compounds

Parameters	Linolic acid	Lupeol	Oleanolic acid
Linearity range (ng/ml)	200-600	200-600	200-600
R_f	0.55	0.62	0.46
Regression coefficient	0.9928	0.9846	0.993
Average	10985.42	6847.67	12283.6
Standard deviation	3320.24	1956.85	3900.34
Standard error	397.37	343.04	148.05
Slop	16.541	09.708	19.494
LOD (µg mL−1)	662.4	665.18	660.26
LOQ (µg mL−1)	2007.4	2015.7	2000.78

Conclusion

The present study showed promising anti-diabetic potential in the plant along with good antioxidant activity. Therefore, it is important to quantify the active secondary metabolites to find out the accurate pharmacological action of respective samples. Hence, the plant can be further explored to be used as a potent therapeutic agent for diabetic disease.

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