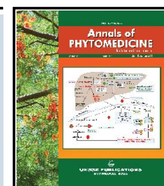
Annals of Phytomedicine 12(2): 562-571, 2023

DOI: http://dx.doi.org/10.54085/ap.2023.12.2.66

Annals of Phytomedicine: An International Journal
http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

HPTLC fingerprinting of quercetin and assessment of *in vitro* and *in vivo* antihyperglycemic effect of the leaves of *Tagetes erecta* L.

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Article Info

Article history
Received 20 September 2023
Revised 4 November 2023
Accepted 5 November 2023
Published Online 30 December 2023

Keywords
Antihyperglycemic
HPTLC
Quercetin
Tagetes erecta L.

Abstract

Tagetes erecta L. also known as marigold, is a foremost plant of Ayurveda to treat different ailments since long. The present study involves qualitative and quantitative estimations, *in vitro* and *in vivo* antihyperglycemic potency and chromatographic separation of quercetin of the leaves of the plant. Five crude extracts of the plants were subjected for qualitative and quantitative estimation along with α-amylase inhibition as per the standard methods. Ethanolic extract was established as more potent candidature for α-amylase inhibition bearing the IC_{50} value of 54.13 mg/ml. Ethanolic extract of the plant in two different doses (100 mg/kg and 200 mg/kg BW) were evaluated for the antihyperglycemic effect on streptozotacin induced diabetic rats. Acute and chronic antihyperglycemic effect of ethanolic extract high dose reduced blood glucose level more significantly. The ethanolic extract of *T. erecta* was subjected for HPTLC fingerprinting using CAMAG LINOMAT 5 instrument in which quercetin was identified.

1. Introduction

Diabetes mellitus, which is characterized by hyperglycemia, is a condition of elevated blood glucose level due to the insufficient pancreatic insulin secretion or contemporaneous impaired actions of insulin (Nolte Martha, 2009). Decrease in circulating concentration of insulin and insulin resistance are thought to be the main causes of the diabetes mellitus. Defect in glucoreceptors of pancreatic β-cell and reduced tissue sensitivity to insulin results in inadequate insulin secretion (Algarsamy, 2014).

Diabetes mellitus, a chronic metabolic disorder, is mainly associated with hyperglycemia, hyperlipidemia, glycosuria and ketonaemia (Tang *et al.*, 2014). Severe complications of hyperglycemia associated with nephropathy, neuropathy, retinopathy, cardiovascular and coronary artery diseases are very common (Papatheodorou *et al.*, 2018). The facts and figures of the current global status of diabetes are frightening and this scenario in SEAR-South East Asia Region is more alarming. As per the report of International Diabetes Federation (2017), in SEAR (India, Bangladesh, Nepal and Sri Lanka), 19.29% of the total diabetic population of the world is living with diabetes and this figure will reach up to 145 million by 2045 (International Diabetes Federation, 2017).

The life-style modifications, physical exercise, medicinal herbs and healthy diet can delay the onset of type 2 diabetes. Medicinal plants and alternative approach towards the management of diabetes are global interest due to the undesired adverse effects of the oral antihyperglycemic agents (Tripathi *et al.*, 2011; Singh and Singh, 2021). Leaves of *Catharanthus roseus, Cocos nucifera, Dillenia indica, Opuntia streptacantha, Symplocos cochinchinensis, etc.*, of antidiabetic potential were reported in earlier literatures (Singh *et al.*, 2015).

In Sushruta Samhita (41st Chapter), it has been mentioned that the marigold plant leaf is slippery in nature and all the benefits of the parts on the basis of principle of *rasa* (taste), *guna* (quality or property), *vipaka* (metabolic effect) and *virya* (potent energy) (Sushrut Samhita, 1989).

T. erecta, belongs to the family Asteraceae, is a plant of enormous therapeutic effect like anthelmintic, antioxidant, antibacterial, cytotoxic, hepatoprotective, analgesic and many more. This plant is mentioned as *Genduk* or *Janduga* in Vedic literatures, whereas, locally it is called as *genda* (in Hindi) and *genda phool* (in Bengali). This plant is annually growing strong and branched herb and widely available in SEAR including India and Bangladesh (Goswami and Singh, 2018; Yasheshwar *et al.*, 2022).

There are so many plants which are mentioned in classics of Ayurveda, but not significantly and scientifically evaluated for the *in vitro* and *in vivo* antihyperglycemic effects. Flower heads of *T. erecta* was a potent α-amylase inhibitor as reported in early study (Hasan and Sultana, 2018). The *in vitro* and *in vivo* antihyperglycemic activity of the crude extract of the leaf part of *T. erecta* and the chromatographic fingerprinting analysis have not been reported yet.

The present research work deals with the *in vitro* and *in vivo* antihyperglycemic activity and HPTLC (high performance thin layer chromatography) fingerprinting analysis of the extract of the leaves of *T. erecta*.

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2. Material and Methods

2.1 Plant collection and authentication

The plant *T. erecta* was collected from Gorakhpur district, Uttar Pradesh, India and the leaves part were identified and authenticated (Specimen No. 03/2017) by ICAR (Indian Council of Agricultural Research)- Kamla Nehru Vigyan Kendra, Sultanpur, Uttar Pradesh, India.

2.2 Preparation of crude extract

The leaves were cleaned to remove foreign matter and were shade dried. The dried leaves were grinded to coarse powder and passed through sieve No. 40. The crude extract was prepared by continuous hot percolation process and cold maceration process with different solvents in increasing polarity. Soxhlet apparatus was used to prepare hot percolated extracts from petroleum ether (TEPE), chloroform (TECE), acetone (TEAcE) and ethanol (TEEE), whereas aqueous extract (TEAE) was prepared from cold maceration process.

2.3 Preliminary phytochemical screening

The crude extracts were subjected for the preliminary phytochemical screening for the alkaloids, tannins, flavonoids and phenolic compounds. Determinations of phytochemical compositions were performed by following the standard methods mentioned in different literatures (Khandelwal, 2008; Falcão and Araújo, 2011; Jadon and Prakash, 2012).

2.4 Qualitative test for alkaloids

Alkaloids were tested by the Mayer's reagent (potassium mercuric iodide solution), Dragendorff's reagent (potassium bismuth iodide solution), Wagner's reagent (iodine-potassium iodide solution) and Hager's reagent (saturated solution of picric acid).

2.5 Qualitative test for tannins

Tannins were identified by the ferric chloride test (1% ferric chloride solution), vanillin assay (1% vanillin in ethanol and 10 ml concentrated HCl) and gelatin test (1% gelatin solution containing 10% NaCl solution).

2.6 Qualitative test for flavonoids

To assure the presence of flavonoids, aqueous sodium hydroxide solution (alkaline reagent test), sulphuric acid test and zinc hydrochloride tests were performed.

2.7 Qualitative test for phenolic compounds

Ferric chloride test, lead acetate test and bromine water test were applied to the ethanolic extract for the qualitative test for phenolics.

2.8 Quantitative estimations

2.8.1 Estimation of flavonoids and tannins

Aluminium chloride colorimetric assay and Folin-Ciocalteau assay methods were employed in triplicates for the estimations of flavonoids and tannins, respectively, as per the previous literatures with modifications (Sulaiman and Balchandran, 2012; Mohammed and Manan, 2015). The results were expressed in mg of quercetin and tannic acid equivalents (per g dry weight), respectively. All the samples were analyzed in three-folds.

2.9 *In vitro* antihyperglycemic activity

2.9.1 α-amylase inhibition assay

Standard procedures (Chelladurai and Chinnachamy, 2018; Nickavar and Yousefian, 2009; Mehrotra *et al*., 2019) were referred for the evaluation of *in vitro* antihyperglycemic activity of all five extracts of the plant leaf part with minor modifications. The α-amylase inhibition assay was performed spectrophotometrically using starch azure-DNS (3, 5-dinitrosalicylic acid) colour reagent method. Sodium phosphate buffer of 0.02 M (pH 6.9) with 0.0067 M NaCl was used in the process. Sample (plant extracts) were diluted in DMSO to attain various concentration ranges from 10 to 100 mg/ml. 1 ml of enzyme solution, which was prepared by dissolving 0.001 g of α-amylase in phosphate buffer (pH 6.9) with the final volume of 100 ml, was added to 1 ml of sample solution, followed by the incubation at 25°C for 5 min 1 ml of starch solution (0.5% w/v) which was prepared in 50 ml buffer solution at 65°C for 15 min was added further to the sample solution and incubated for 3 min at 25°C. To this solution 1 ml of DNS spectrometric colour reagent was added and the whole reaction mixture was placed on water bath for 15 min, followed by cooling and adding of 9 ml of distilled water.

Blank solution was prepared by omission of enzyme solution by the phosphate buffer and adding of DNS colour reagent prior to the starch solution. By taking DMSO in place of sample extract was followed to prepare the control solution.

For the *in vitro* assay, acarbose was considered as standard.The absorbance of standard, sample, blank and control were measured at 540 nm in double beam UV visible spectrophotometer (Systronics India).

The following formula was used in the calculation of α-amylase inhibition:

$$\alpha\text{-amylase inhibitory activity (\%)} = \frac{(A_{control} - A_{sample\,extract})}{A_{control}} \times 100$$

where A= Absorbance

2.10 Statistical analysis

The results were analyzed in triplicate (n=3); results were communicated as ± standard error of the mean (SEM); analysis was done by one-way ANOVA followed by Tukey's multiple comparison test and graphs were drawn in Graph Pad Prism 7.4 and Origin 6.1; ^{a} p<0.0005 and ^{b} p<0.0001 were considered as significant.

2.11 *In vivo* antihyperglycemic activity

2.11.1 Plant extract

TEEE, ethanolic extract of *T. erecta* of different doses was selected for the experiment.

2.11.2 Animals used

Healthy albino rats (140-160 g weight) were selected for the study and kept for 1 week prior to initiation of experiment in animal house at room temperature on a cycle of 12 h of light and 12 h of dark with proper arrangement of diet and water. After the approval

of Institutional Animal Ethics Committee (IEAC) of Suresh Gyan Vihar University (Vide approval No: SGVU/PH/IAEC/2017/01), the experiments were conducted.

2.11.3 Induction of diabetes

Induction of diabetes was done in overnight fasted healthy selected rats by intraperitoneal injection of streptozotacin (STZ) at the dose of 50 mg/kg body weight (BW) with sodium citrate buffer (0.1M). More than 200 mg/dl of fasting blood glucose level (after 48 h of administration) in rats confirmed the diabetes induction and those were further selected for the experiments. The doses of the TEEE were selected by the acute toxicity study as per the OECD guideline. Glibenclamide (GLI), a well-known antidiabetic drug, was considered for standard treatment (Tripathi *et al*., 2013).

2.11.4 Treatment protocol

For the evaluation of the antihyperglycemic activity, the following five (n=5) treatment protocols were designed (Goswami and Singh, 2019):

Group A: Non-diabetic control; denoted as NC

Group B: Diabetic control; denoted as DC

Group C: Diabetic rats treatment with GLI at the dose of 0.5 mg/kg BW and noted as DC + GLI.

Group D: TEEE in low dose (100 mg/kg BW); mentioned as DC + TEEE (100 mg/kg)

Group E: TEEE in high dose (200 mg/kg BW); mentioned as DC + TEEE (200 mg/kg)

2.11.5 Estimation of blood glucose

For the evaluation of acute effect of TEEE, blood glucose level was verified in every 2 h up to 6 h and in 24 h. Chronic study was meant for long term effect, so, the glucose level was checked for a period of 28 days at the regular interval of one week (Pandey *et al*., 2013). The blood samples were collected from tail-vein of all rats and the blood glucose level (in mg/dl) was checked instantly by glucometer (Accu-chek, Germany).

2.11.6 Change in body weight

The effect of plant extracts on BW of rats was examined before commencement (Day 0) and after completion (Day 28) of the study.

2.11.7 Statistical evaluation

The results were expressed as mean as ± standard error of the mean (SEM); statistical evaluation were analyzed with the help of Graph Pad Prism 7.4 software; analysis was done by one-way ANOVA followed by Tukey's multiple comparison test and Dunnett's multiple comparison test; as compared to the diabetic group $^{b}p<0.01$ and $^{a}p<0.001$ were considered as significant.

2.12. HPTLC fingerprinting

2.12.1 Plant extract

HPTLC studies of TEEE were performed according to the standard method (Goswami and Singh, 2019; Varghese *et al*., 2013; Shwetha *et al*., 2020).The apparatus, used for the HPTLC fingerprinting, was CAMAG LINOMAT 5 instrument (Camag; Muttenz, Switzerland, WINCATS software). Quercetin was considered as standard. 1 ml HPTLC grade ethanol was used to dissolve 100 mg TEEE followed by centrifugation. The TLC twin trough developing chamber was saturated for 25 min at room temperature with the selected mobile phase of toluene, ethyl acetate and formic acid in the ratio 13:11:2.2 µl each of standard and sample solutions were applied by LINOMAT 5 automated applicator with a 100 µl Hamilton syringe on 3 × 10 cm TLC plate (Silica gel 60 F_{254}) as bands of 5 mm. The plate loaded with sample was kept in saturated developing chamber.The chromatogram was developed in the twin trough developer up to 80 mm solvent front from the point of sample application and dried in hot air oven to remove the solvent. In photo-documentation chamber (CAMAG REPROSTAR 3), the plates were kept and images were captured at two different wavelengths, 254 nm and 366 nm. The plates, after derivatization, were placed in CAMAG TLC SCANNER 3 for densitometric scanning. All the results were noted.

3. Results

3.1 Preliminary phytochemical screening and quantitative estimations

Phytochemical tests of all the extracts of *T. erecta* were reported in Table 1 and the presence of tannins, phenolic compounds and flavonoids were confirmed in all the extracts. Alkaloids were absent in TEPE and TECE, whereas, flavonoids were more abundant in TEEE and TEAE.

Table 1: Phytochemical screening of *T. erecta*

Phytochemicals	TEPE	TECE	TEAcE	TEEE	TEAE
Alkaloids					
Mayer's test	-	-	-	+	-
Dragendorff's test	-	-	-	+	-
Wagner's test	-	-	+	-	-
Hager's test	-	-	-	+	+
Tannins					
Ferric chloride test	-	-	-	+	-
Vanillin test	-	+	+	+	+
Gelatin test	+	+	+	+	+

Phenolic compounds					
Ferric chloride test	-	-	-	+	-
Lead acetate test	+	+	-	+	+
Bromine water test	-	-	+	+	+
Flavonoids					
Alkaline reagent test	-	-	-	+	+
Sulphuric acid test	+	+	-	+	+
Zinc hydrochloride test	+	+	+	+	+

(+ indicates 'Presence' and – indicates 'Absence' of phytoconstituents).

Quantitative estimation of tannins and flavonoids were represented in Figure 1. Total tannin and total flavonoids were found more in TEEE (4.05 ± 2.15 mg TAEg, 21.80 ± 2.01 mg QEg), while compared to TEAE (1.24 ± 1.24 mg TAEg, 15.11 ± 0.08 mg QEg) and TEAcE (1.01 ± 3.41 mg TAEg, 7.14 ± 2.41 mg QEg), whereas, the amount was very less in TEPE and TECE.

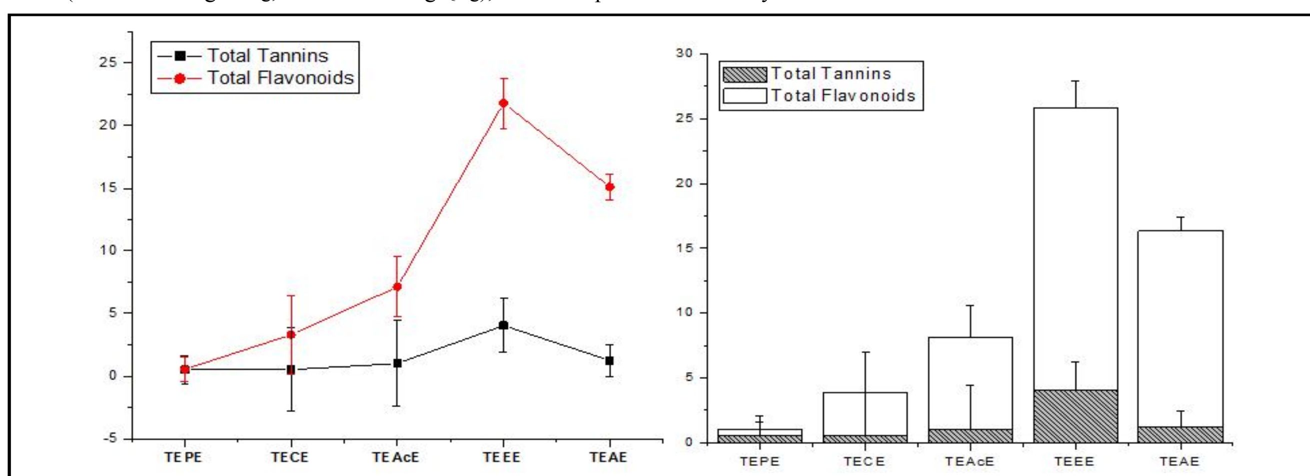

Figure 1: Quantitative estimations of *T. erecta*, all values represent mean ± SD; triplicate (n=3).

3.2 α-amylase inhibition assay and IC_{50} value

The α-amylase inhibition assay of all five extracts was represented in Figure 2 and the result was dose dependent. Significant inhibition was observed for all the extracts, moreover, TEEE represented itself as potent inhibitor of α-amylase. TEEE, at higher concentration (60-100 mg/ml), was significantly (p<0.0001) different from other extracts.

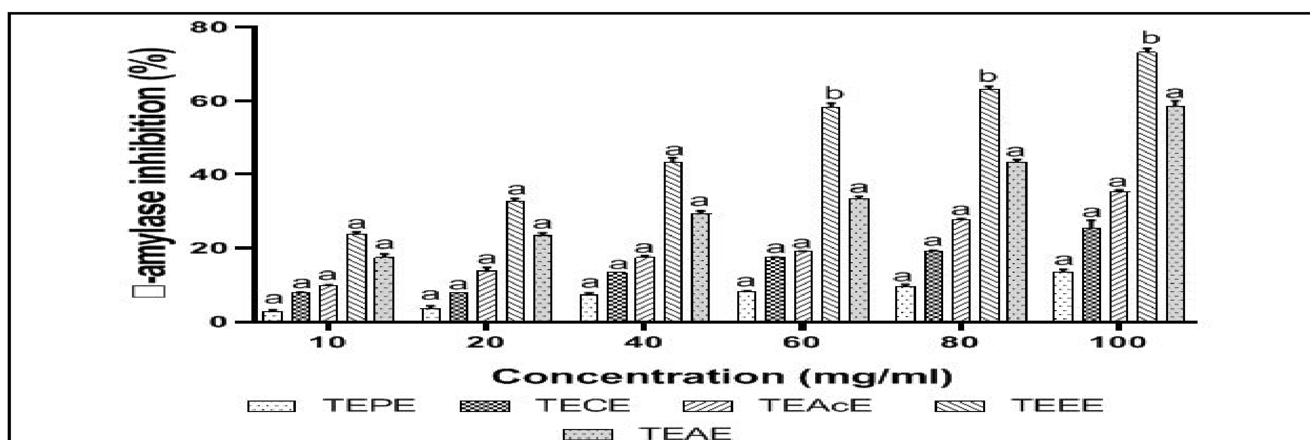

Figure 2: α-amylase inhibition of *T. erecta* leaf extracts. Results were represented as ± SEM; Analysis was done by one-way ANOVA followed by Tukey's multiple comparison test; means not sharing common letter in the same concentration was more significant (*p<0.0001*).

At 100 mg/ml TEEE showed highest inhibition (73.23 ± 1.06) than TEAE (58.65 ± 1.38); whereas, acarbose, the positive control, also showed the inhibition in dose dependent manner and the maximum inhibition of 76.34 ± 0.47 was observed at 100 µg/ml (Figure 3).

IC_{50} value result (Figure 4) revealed that TEEE (54.13 mg/ml) and TEAE (90.19 mg/ml) had the lower value as compare to other extracts. Standard drug, acarbose, showed the IC_{50} value as 61.06 µg/ml.

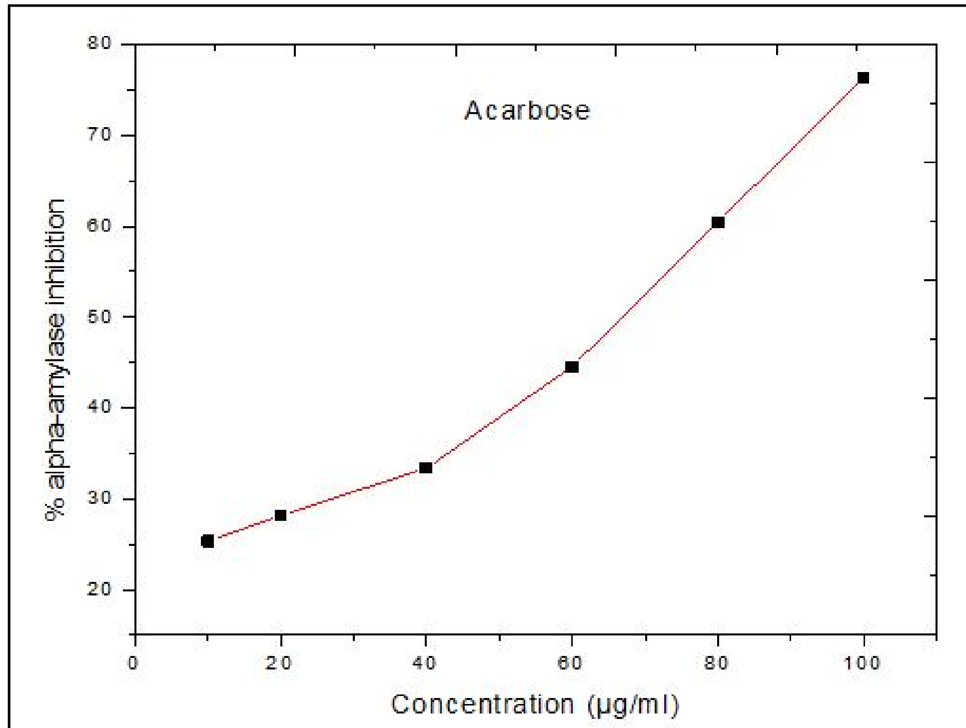

Figure 3: % α-amylase inhibition of different concentrations of acarbose.

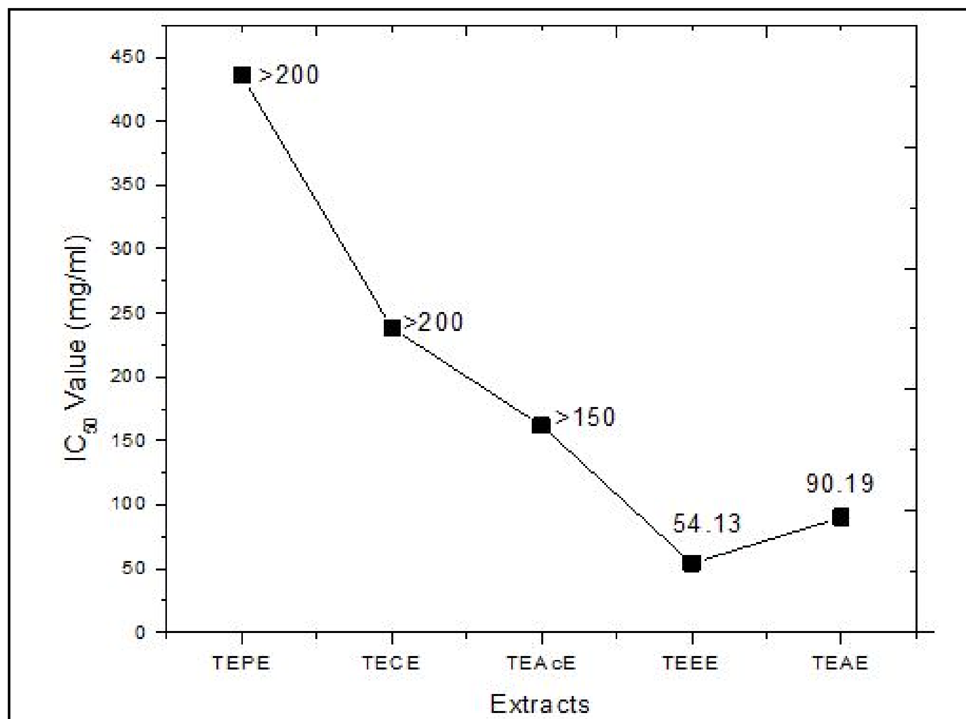

Figure 4: Comparative IC_{50} value of extracts of *T. erecta* leaf.

3.3 *In vivo* antihyperglycemic activity

The result of acute toxicity study was satisfactory and up to a dose level of 2000 mg/kg body weight, the ethanolic extract of the plant was safe. Antihyperglycemic effect of *T. erecta* was determined by acute and chronic study in two different doses (100 and 200 mg/kg) of TEEE and significant activity was observed. In the first day, the blood glucose levels were checked at different time intervals up to 24 h to assess the acute effect of the extract (Figure 5). The antihyperglycemic effect of the standard drug (DC + GLI) was more intense (20.90%) than low dose (11.02%) and high dose (16.85%) of DC + TEEE. The chronic effect was evaluated by monitoring the blood glucose level at the interval of 7 days and the follow up was continued for 28 days (Figure 6). At the end of the study, standard drug treatment exhibited significant and highest antihyperglycemic activity comparing to acute effect (57.83%), whereas, DC + TEEE (200 mg/kg) reduced the blood glucose from 261 ± 2.7 mg/dl (0 day) to 141 ± 2.77 mg/dl (28th day) which is more effective than DC + TEEE - 100 mg/kg (263 ± 5.1 mg/dl to 170 ± 3.71 mg/dl). Figure no 7 represented the comparative significant activity (one-way ANOVA followed by Dunnett's multiple comparison test) against diabetic control of TEEE high and low dose.

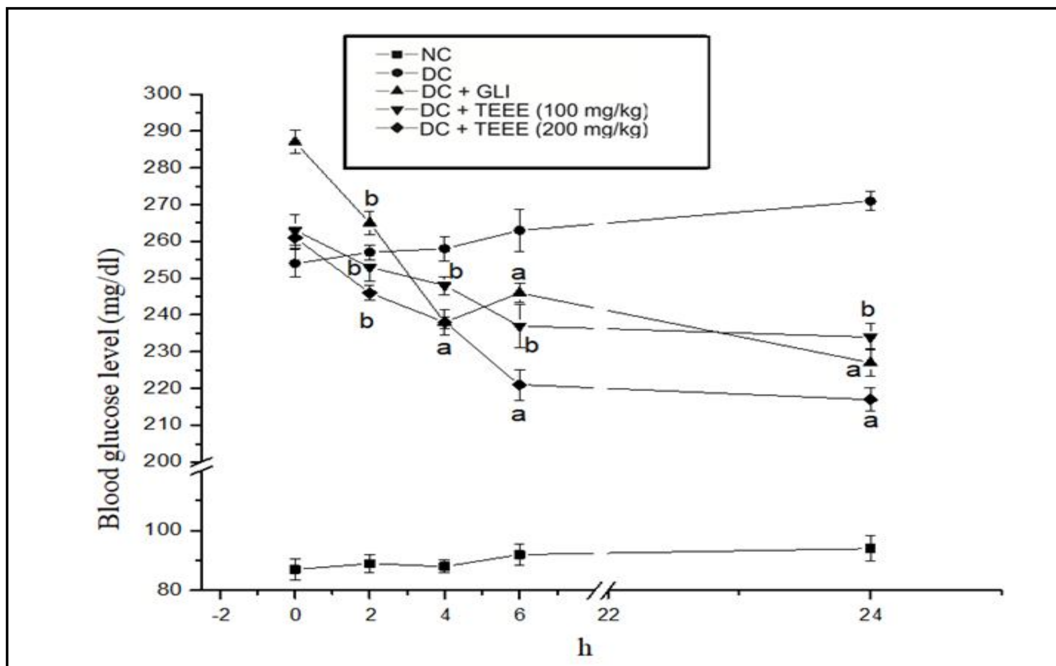

Figure 5: Acute effect of TEEE on hyperglycemia. All values were expressed as ± SEM (n=5); analysis was done followed by Tukey's multiple comparison test; different letters represented different significant levels.

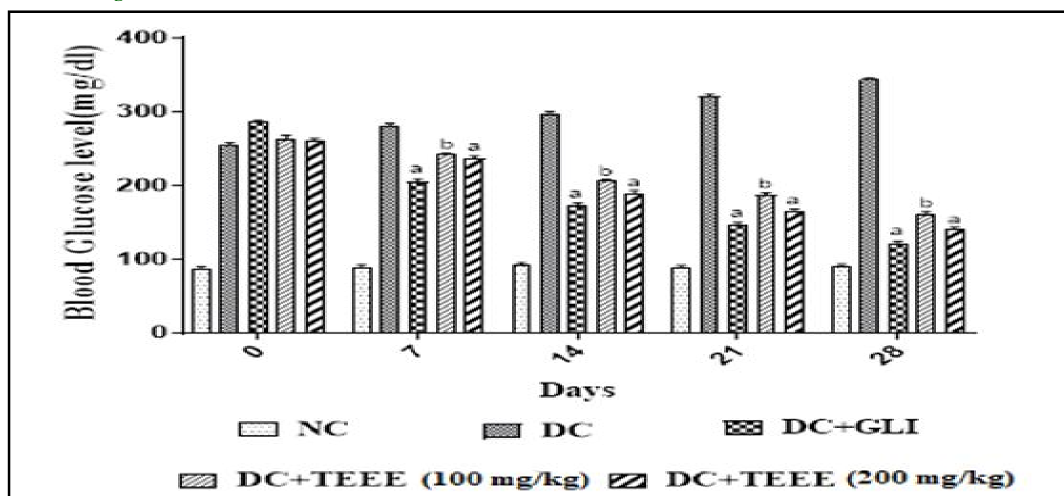

Figure 6: Chronic effect of TEEE on hyperglycemia. All values were expressed as ± SEM (n=5); analysis was done followed by Tukey's multiple comparison test; means sharing common letter in the same concentration was more significant ([b] *p<0.01* and [a] *p<0.001)*.

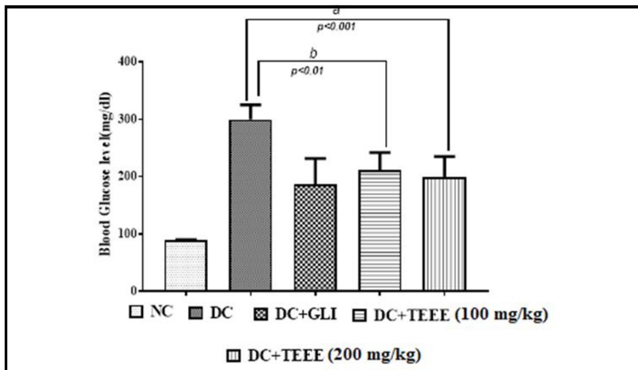

Figure 7: Significant chronic antihyperglycemic effect of TEEE against diabetic control. All values were expressed as ± SEM (n=5); analysis was done by one-way ANOVA followed by Dunnett's multiple comparison test; [b] $p<0.01$ and [a] $p<0.001$ were considered as significant.

The effect of the standard and the extracts on body weight of the animal was studied and monitored throughout the study. The percentage change in body weight of the animals mentioned in Figure 8 and the weight change in group C, D and E was reported as 7.95, 11.68 and 12.05, whereas, the weight of the animals belongs to group B decreased by 13.26%.

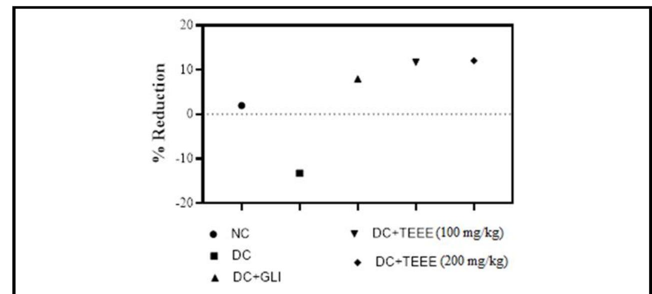

Figure 8: Effect of TEEE on body weight.

3.4 HPTLC fingerprinting

The chromatographic analysis (HPTLC) of TEEE was performed for the isolation of phytoconstituents with suitable solvent system. HPTLC chromatogram profile was given in Figure 9 which was visualized under UV 254 and 366 nm. HPTLC densitogram (Figure 10) revealed 15 bands in which sixth band was identified as quercetin while compared to standard (Figure 11).

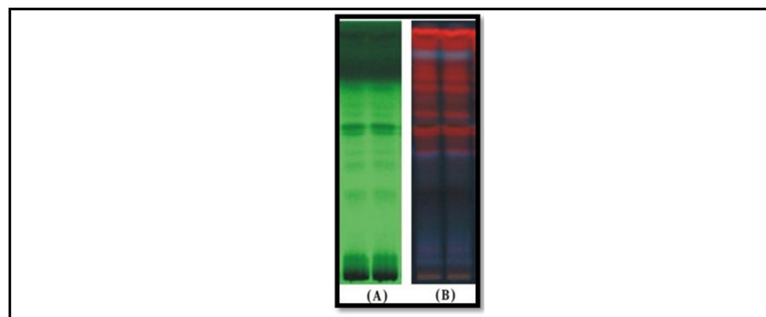

Figure 9: HPTLC chromatogram of TEEE; (A) UV 254 nm and (B) 366 nm.

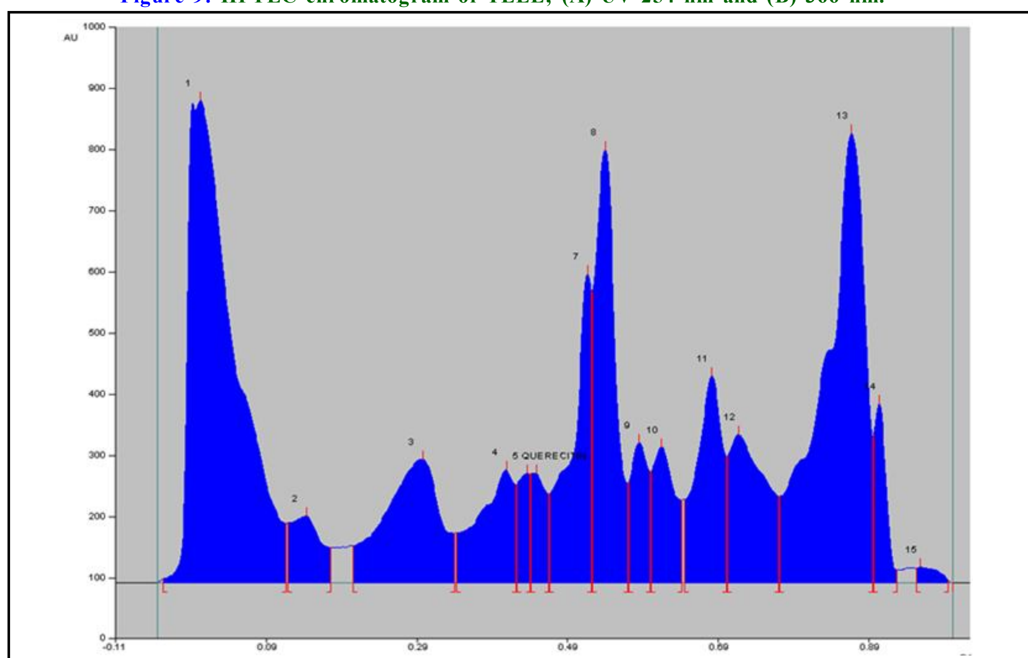

Figure 10: HPTLC densitogram of TEEE.

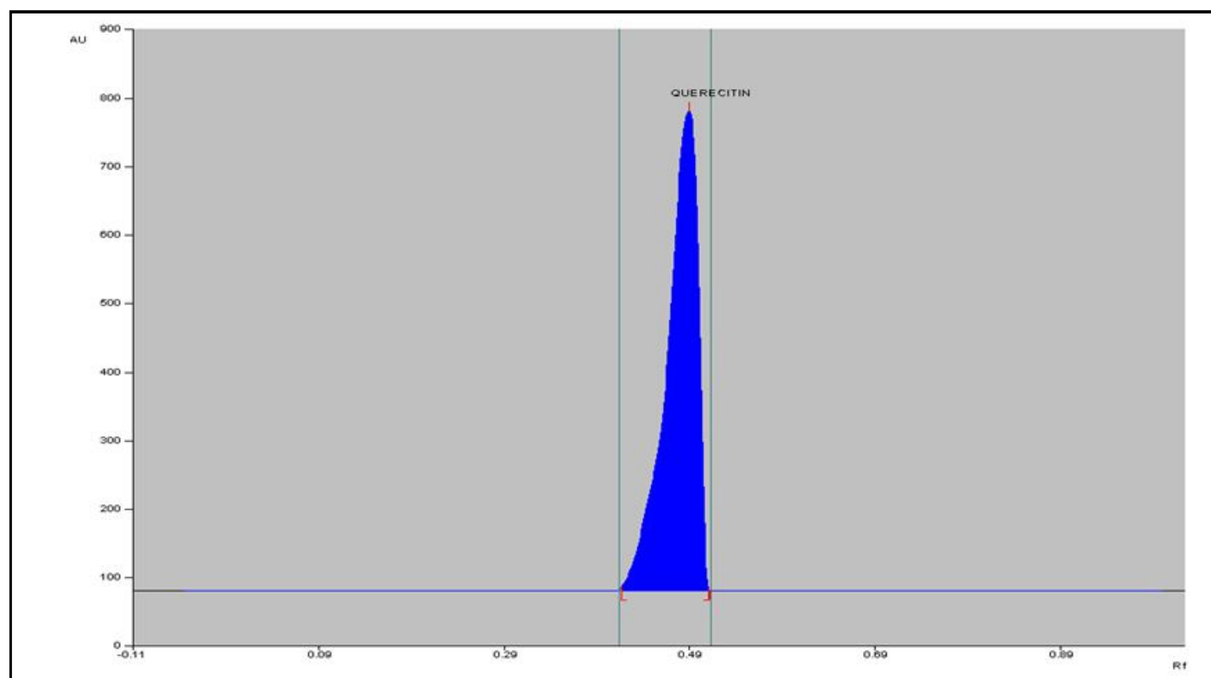

Figure 11: HPTLC densitogram of standard quercetin.

R_f values, peak heights and peak areas of standard and isolated sample were tabulated in Table 2.

Table 2: HPTLC profile of TEEE with R_f values

Track name	R_f	Max Height (%)	Area (%)	Assigned substances	Remark
T. erecta	0.46	3.62	1.83	Flavonoid (quercetin)	Band No 6
Standard	0.51	100	100	Quercetin	Standard

4. Discussion

Diabetes is a metabolic disorder associated with the alteration of carbohydrate, fat and protein metabolism. Insulin is secreted from pancreatic cells for the regulations of blood glucose either by increasing the glucose uptake into liver and muscles or by the inhibition of gluconeogenesis and glycogenolysis. Continuous stagnation of β cells which diminish the insulin secretion and insulin resistance combinedly cause hyperglycemic condition. Insulin resistance though creates a hyperinsulinemic stage for a while, but causes diminution of utilization and uptaking of glucose, and ultimately affects phosphorylation of insulin receptor substrate (IRS) and translocation of glucose transporters (GLUT-4) (Zito, 2013). To eliminate the severe diabetic complications of synthetic drugs, safest and cost-effective approach towards alternative medicines especially various phytoconstituents are the present interest for the researchers (Gupta, 2018).

Phytoconstituents like alkaloids, flavonoids, tannins and phenolics possess antihyperglycemic action by the effect either on carbohydrate digestion, absorption and glucose transportation or insulin secretion. These phytoconstituents have significant effect also on the enzyme, α-amylase (Bharti, 2018; Mounika and Hymavathi, 2021). The present investigation of phytochemical tests confirmed the presence of all the above mentioned constituents in TEEE and TEAE, whereas, alkaloid in TEPE and TECE was absent. Flavonoids, tannins and phenolics were observed in all extracts including TEPE and TECE. Total tannins and total flavonoids were quantified more in TEEE comparing to others.

In type 2 diabetes, one of the important treatment strategies is decreasing the postprandial blood glucose level and that can be achieved by the inhibition of α-amylase enzyme (Sun *et al*., 2017). The enzyme is responsible for the breakdown and hydrolysis of starch into smaller units resulting in hyperglycemia (Shankaraia and Reddy, 2011). The inhibitors of the enzyme postpone the absorption of the glucose and extend the absorption time, hence help to treat the condition of postprandial glucose level (Sales *et al*., 2012).The present research established the *T. erecta* as a potent á-amylase inhibitor and the effect was concentration dependent. Similar studies also reported by showing prominent inhibitory effect of *Salacia oblonga* stem (Chelladurai and Chinnachamy, 2018) and *Gossypium arboretum* (Kazeem *et al*., 2013) on α-amylase were reported. The order of the inhibiting effect of all the extracts in the present study was TEPE<TECE<TEAcE<TEAE<TEEE. At 100 mg/ml TEEE was significantly (^{b} *p<0.0001)* different from others. The amylase inhibitory effect of *Schleichera oleosa* Oken was evaluated by the authors earlier and ethanolic extract was reported as more significant among others (Goswami and Singh, 2018).

The belief on medicinal plants as more safe than the synthetic drugs the people are switching to herbal drugs from synthetic drugs (Karimi, 2015).The present evaluation also reported the *in vivo* antihyperglycemic activity of TEEE in STZ induced diabetic rats. The TEEE was more potent in α-amylase inhibition, hence selected for the *in vivo* activity. The results were significant as compared to diabetic control. GLI, a class of sulfonyl urea oral antihyperglycemic, is believed to be effective in hyperglycemia as it promotes insulin secretion from pancreatic β cells (Rambiritch *et al*., 2014). In acute study, the blood glucose reduction of GLI treated rats in first 2 h was more potent (7.66%) than TEEE high dose (5.75%) and low dose (3.80%). The effect was continued for first 24 h and the inhibition of blood glucose of TEEE higher dose was far better than lower one. The similar reports were mentioned for *Kyllinga triceps* (Lal *et al*., 2012) and *Rhizophora mucornata* (Pandey *et al*., 2014) plant extracts. For long term treatment up to 28 days, TEEE 200 mg/kg (45.97%) effect was more potent than 100 mg/kg dose (35.36%) while compared to standard (57.83%). The similar effect was reported in aqueous extract of *Trixis angustifolia,* where 100 mg/kg was more antihyperglycemic than 50 mg/kg dose (Salazar-Gómez *et al*., 2019). Obesity is one of the key aspects in the treatment of type 2 diabetes. Traditional antihyperglycemic medicines tend to increase the body weight (Gaal and Scheen, 2019). So, the follow up of BW of the animals throughout the study carried out.

Isolation of quercetin from plant extracts and their antihyperglycemic activity was reported earlier (Dureshahwar *et al*., 2017). In the present research chromatographic isolation of TEEE was done by HPTLC in which quercetin was identified along with other unknown flavonoids. The potential antihyperglycemic effect of quercetin was multifaceted and shows effect on insulin secretion, sensitization and glucose absorption (Eid and Haddad, 2017).

5. Conclusion

Type 2 diabetes and its incidence have profound health consequences. Oral antihyperglycemic agents have shown to exert several disadvantages including drug resistance and toxicity. Herbal treatment lines have been therefore implicated as an alternate traditional system towards management and prevention of diabetes. Advances in field of traditional system of medicines have fuelled inclusion of medicinal plants for therapeutical interventions. The present work revealed *T. erecta* as a potent antihyperglycemic which support its traditional and folkloric use. The isolation and characterization of active constituents related to quercetin, responsible for the antihyperglycemic activity and the molecular level mode of action will be the future interest.

Acknowledgements

The authors are grateful to the research and management committee of NIMS University, Jaipur, Rajasthan and Divine College of Pharmacy, Bihar, India for their support and encouragement in conducting this research work and publishing the reports.

Conflict of interest

The authors declare no conflict of interest relevant to this article.

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Citation **Ravindra Pal Singh,Shambaditya Goswami and Soumya Pathak (2023). HPTLC fingerprinting of quercetin and assessment of *in vitro* and *in vivo* antihyperglycemic effect of the leaves of *Tagetes erecta* L.. Ann. Phytomed., 12(2):562-571. http://dx.doi.org/10.54085/ap.2023.12.2.66.**