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Molecular Docking and Potential Therapeutic Effect of *Chrysanthemum indicum* on Streptozotocin Induced Diabetic Neuropathy in Rodent Models

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ABSTRACT

Neuropathy, a common complication of diabetes mellitus, is generally considered to be related to duration and severity of hyperglycaemia. Chrysanthemum indicum belonging to family Asteraceaeis medicinally reported to cure various diseases in Indian traditional system (Ayurveda) and in folklore. The purpose of this study is to explore diabetic neuropathy in streptozotocin induced diabetes using methanolic extract of Chrysanthemum indicum flowers and the study attempts to establish a relationship between ethnopharmacological claims and bioactive constituents present in Chrysanthemum indicum against all possible targets for diabetes through molecular docking and to develop a pharmacophore model for theactive target. Gabapentinis used as standard drug. Pharmacological evaluations of MECI were carried out using 200 and 400 mg/kg, bd.wt.in normal and diabetic rats. After 2 weeks of STZ administration there was a significant increase in serum glucose and other biochemical parameters like MDA levels in sciatic nerve as well as mechanical allodynia indicating hyperalgesia. Sciatic nerve estimations revealed increase in lipid peroxidation level, and a decrease in antioxidant parameters. The MECI significantly decreased blood glucose level in normal and diabetic rats. A dose of 400 mg/kg significantly increases the tail withdrawal latency time with hot and cold water tail immersion tests respectively. The process of molecular docking involves study of different bonding modes of one ligand with active cavities of target receptors of protein kinase inhibitor (PDB ID: 2I0E), an aldose reductase inhibitor (AR)(PDB ID: 4GCA), and PPAR γ agonist (PDB ID: 1FM6) using Schrodinger Software Maestro 9.1.Docking studies revealed score values on different receptors for antidiabetic activity and it is observed that constituents namely, syringaresinol, 5,6,7-trihydroxy 3,4,5' trimethoxyflanon, tricin and quercetin showed the best docking results on almost all the receptors, while the most significant results were observed by syringaresinol, 5,6,7-trihydroxy 3,4,5' trimethoxyflanon and tricin on PPAR-γ agonist (PDB ID: 1FM6). Thedocking results have given better insights in the development of better PPAR-γ so as to treat diabetes relatedsecondary complications.From the results MECI possesses potential active constituents to combat glucose suggesting its role in preventing diabetes–related complications like neuropathy.

KEYWORDS: *Hyperglycemia, Streptozotocin, PPAR-γ, Malondialdehyde and Gabapentin.*

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INTRODUCTION

Diabetes Mellitus (DM) is probably one of the oldest diseases known to man. Type 2 DM is most common *i.e.,* characterized by hyperglycemia, insulin resistance, and relative insulin deficiency that results from interaction between environmental, genetic and behavioural risk factors [1]. Neuropathy, a common complication of diabetes mellitus, is generally considered to be related to duration and severity of hyperglycaemia. Diabetic neuropathy has been defined as presence of symptoms or signs of peripheral nerve dysfunction in diabetes after exclusion of other causes which may range from hereditary, traumatic, compressive, metabolic, toxic, nutritional, infectious, immune mediated, neoplastic, and secondary to other systemic illnesses. Since the manifestations of diabetic neuropathy closely mimic chronic inflammatory demyelinating polyneuropathy, alcoholic neuropathy, and other endocrine neuropathies, hence before labelling diabetic neuropathy it is mandatory to exclude all other causes of peripheral nerve dysfunction.

Herbal medicines are popular as remedies for diseases and play a key role in human health care of a vast majority of world's population [2].*Chrysanthemum indicum L* or *Dendranthema indicum,* belongs to family *Asteraceae* (Compositae) is native to India, China, Bangladesh, European countries. The flowers are used for antibacterial action, furuncle, scrofula, deep-rooted boils, inflammation of the throat, eyes and cervix,

eczema, itchiness of the skin and hypertension [3].The aim of the study is to investigate the effect of *Chrysanthemum indicum* Linn on streptozocin induced diabetic neuropathy in rodent model.

MATERIAL AND METHODS

Plant collection

The flowers of *Chrysanthemum indicum* were collected from Bachannapet village, Jangaon district, Telangana in the month of January and were authenticated. The flowers were cleaned, reduced to small fragments, dried under shade for about six days and coarsely powdered in a mixer grinder. The powdered material was stored or taken up for extraction process.

Preparation of methanolic flower extracts *Chrysathemum indicum*

Soxhlet extraction is the process of continuous extraction in which the same solvent can be circulated through the extractor for several times. This process involves extraction followed by evaporation of the solvent. The vapours of the solvent are taken to a condenser and returned to the drug for continuous extraction. The organic extracts obtained were evaporated to dryness by keeping at room temperature [4].

Preliminary Phytochemical Investigation of Plant extract

The methanolic flower extract *Chrysathemum indicum* was subjected to preliminary phytochemical screening to identify various phytoconstituents presence.

Animals

Swiss albino mice (20 to 25 gm) and Wistar albino rats of (250-300 gm) were procured from Albino research laboratories, Hyderabad. The animals were housed in poly acrylic cages with not more than six animals per cage, with 12 h light/12 h dark cycle. The animals were allowed to acclimatize the laboratory environment for a week before the start of the experiment. The care and maintenance of the animals were carried out as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, India. (Reg.No. 1175/PO/ERe/S/08/CPCSEA).

Acute toxicity testing

Acute toxicity studies were carried out in order to check the toxic effects of the methanolic flower extract *Chrysanthemum indicum* as per Organization for Economic Cooperation and Development (OECD) 425 guidelines. The animals were fasted overnight, providing only water after which the extract was administered to the respective groups orally at the dose level of 2000 mg/kg body weight by gastric intubation and the animals were observed continuously for 24 hours for behavioural, neurological and autonomic profiles, and then at 24 h and 72 h for any lethality.

DIABETIC NEUROPATHY

Diabetic neuropathy

Diabetes was induced in rats by single *i.p.* injection of STZ 55mg/kg,bd.wt. in freshly prepared cold citrate buffer (pH 4.5). In order to prevent fatal hypoglycemia due to massive pancreatic insulin release, rats were treated with 15% glucose solution bottles in their cages from 6 h to a period of 24h. After 72h, the animals showing blood glucose level above250mg/dL were considered diabetic and are used for the study. Diabetic rats were kept under standard laboratory condition for the stabilization of blood glucose level during the period of study.

Treatment and experiment protocol

The rats were randomly divided in five groups (n=6), Group 1: Normal control (only saline treated); Group 2: STZ–Diabetic control(55 mg/kg, b.w.); Group 3: rats treated with 200 mg/kg, b.w. of MECI; Group 4: rats treated with 400 mg/kg, b.w. of MECI; Group 5: rats treated with gabapentin3.6 mg/kg, b.w. After six weeks of induction of diabetes in animals, Methanolic extract of MECI administered to animals for 14 days (during 7^{th} to 8^{th} week).Every day the dose of the extract was freshly prepared and a dose of 200 and 400 mg/kg was given to group III and IV and a dose of 3.6mg/kg of gabapentin to the group V. Tail flick latency, was measured at 7^{th} and 8^{th} week with cold and hot water tail immersion. Paw licking was measured using hot plate method. At the end of the experiment, animals were sacrificed for the study of biochemical changes [5].

Behavioural studies

Thermal hyperalgesia

Thermal stimuli: the tail immersion test – warm 45°C

The tail of the rat was immersed in a water bath at a temperature of 45°C until tail withdrawal or signs of struggle were observed (cut-off time: 15 set).

Thermal stimuli: the tail immersion test – cold 10°C

The tail of the rat was immersed in a water bath at a temperature of 10°C,a temperature that is normally innocuous. Shortened duration of immersion indicates allodynia. The cut-off time was 15 sec [6].

Hot plate method 54°C

The hot plate latency was measured using a modification of the original method. Briefly, the modified apparatus consists of an electric cooking plate with a 1500 Watts stainless steel heating element connected to a thermostat (0-40°C); a thermocouple connects the thermostat to a chrome plated drip pan. The thermocouple together with the thermostat control the temperature of the hot plate within the desired range once set. Pain sensitivity was evaluated by the response latency for paw licking on the hot place. In order to avoid tissue damage, the maximum time the animal could spend on the hot plate was 15 seconds [7].

Estimation of biochemical parameters

Lipid peroxidation MDA assay:

The MDA level, an indicator of free radical generation, was estimated in the sciatic homogenate in a ratio of 1 g tissue to 9 mL potassium phosphate (50 mM) plus EDTA (0.1 mM) buffer, pH 7. The lipid peroxide level was determined using the thiobarbituric acid test. Briefly, 0.2 mL of homogenate was added to 0.8% thiobarbituric acid, 8.1% sodium dodecyl sulfate (SDS) and acetic acid (20%) in distilled water. After heating for 60 min in a water bath at 95 ℃, the mixture was then cooled and extracted with a mixture of *n*-butanol/pyridine (15:1v:v). The absorbance of the reaction product present in the upper organic layer separated by centrifugation was measured spectrophotometrically at 532 nm [8].

Docking Studies

The three-dimensional crystal structure of different receptors taken from Protein Data Bank (PDB) (http://www.rcsb.org/) is as follows: PKC (PDB ID: 2I0E), AR (PDB ID: 4GCA), PPAR γ (PDB ID: 1FM6). All the PDB's were loaded in the Schrodinger with the removal of all water molecules. The retrieved structures were pre-processed in Schrödinger Maestro. This step included simplification of multimeric structures, proper assignment of bond orders and ionization states, addition and optimization of hydrogen bonds, location and deletion of unnecessary water molecules, creation of disulphide bonds, conversion of selenomethionines to methionine, aligning and capping of terminal amides, addition of missing atoms and side chain residues, and assignment of partial charges. Finally, restrained minimization was performed to obtain a geometrically stable structure [9].

Statistical analysis

Values are expressed as Mean ± SEM, (n=6). All the groups were compared with control, diabetic control and standard by using Dunnett's test.

RESULTS

Methanolic flower extract of *Chrysanthemum indicum* was screened for its diabetic neuropathy. All the results obtained in this study were included below.

Preparation of methanolic extract of *Chrysanthemum indicum*

The methanolic extract of flower of *Chrysanthemum indicum* was prepared by soxhalation technique. The percentage yield of the extract was calculated by using the following formula

% yield of extract = Amount of extract obtained / Amount of powder used X 100

= 60/50 * 100= 12% w/w.

Preliminary phytochemical screening

Methanolic flower extract of *Chrysanthemum indicum* showed the presence of Saponins, flavonoids, phenolic compounds, steroids, triterpenoids, glycosides, carbohydrates proteins and volatile oils. The results are showed in table 1.

Table 1:Preliminary phytochemical analysis.

Phytochemical constituents	Results
Flavonoids	++
Phenolic compounds	++
Steroids	++
Triterpenoids	+
Glycosides	+
Carbohydrates	+
Volatile oils	+
Saponins	-

Note: + indicates present; - indicates absent.

ACUTE TOXICITY STUDIES:

Acute toxicity studies of methanolic extract of *Chrysanthemum indicum* were performed as per OECD guidelines 425,did not exhibit any signs of toxicity and mortality even upto 2000 mg/kg. bd. wt. All animals were safe even after 14 days of observation.

***In-vivo* anti-diabetic activity**

The methanolic extract of *Chrysanthemum indicum* was tested to determine its effects on blood glucose level and body weight streptozotocin induced diabetic rat model.

Diabetic neuropathy

Behavioural studies* (*Effect of extract on thermal hyperalgesia)

Effect of two week treatment with MECI (200 & 400 mg/kg) on various behavioural parameters in diabetic neuropathy.

Table2:Effect of MECI on hyperalgesia in STZ induced diabetic neuropathic activity

Treatment	Tail immersion method hot (sec)		Tail immersion method cold(sec)		Hot plate method Paw licking (sec)	
	7th week	8th week	7th week	8th week	7th week	8th week
Normal Control	14.33±0.19	14.3±0.19	14.1±0.15*	14.5±0.2	13.66±0.3*	14.5±0.2
Negative Control (STZ 55 mg/kg)	5.46±0.20*	3.8±0.15*, a	5.16±0.2*	4.6±0.1*, a	5.±0.2*	3.83±0.28*, a
MECI (200 mg/kg)	5.66±0.19*	11.5±0.2*, A, a	6.66±0.19*	10.1±0.15*, A, a	5.16±0.15*	9.3±0.19*, A, a
MECI (400 mg/kg)	6.83±0.15*	12.16±0.28*,A, a	5.5±0.2*	11.5±0.2*, A, a	5.3±0.19*	10.6±0.19*, A
Gabapentin (3.6 mg/kg)	5.43±0.30*	13.6±0.19*, A,	5.3±0.3*	13.6±0.19*,A	5.5±0.2*	12.16±0.15*, A

Values are expressed as Mean±SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were compared with normal control group (*=p<0.01), diabetic control (A= p<0.01) and standard (a = p< 0.01), ns- non significant.

By the above results the pain scores in diabetic rats were significantly decreased due the development of hyperalgesia. But after the treatment with methanolic extract of *chrysanthemum indicum* at 200 mg/kg and 400 mg/kg the hyperalgesia was reversed and pain scores were increased when compared to diabetic rats.

Biochemical parameters

Estimation of blood glucose levels

Table 3: Effect of MECI on blood glucose levels

Treatment	Blood glucose level (mg/dL)	
	1st week	8th week
Normal Control	89.667±0.608	86.333±0.561
Negative Control (STZ 55 mg/kg)	300.83± 0.390	313.83±0.280
MECI(200 mg/kg)	305.33±0.3849	200.83±0.495
MECI(400 mg/kg)	310.33±0.304	180.67±0.509

From the above results it is clear that the blood glucose levels of the diabetic rats were elevated when compared with normal rats. Upon treatment with methanolic extract of flower of *Chrysanthemum indicum* at 200 mg/kg. bd. wt, 400 mg/kg. bd. wt. significantly reduced the elevated blood glucose levels of diabetic rats.

Lipid peroxidation of sciatic nerve

Estimation of nerve MDA levels

Table 4: Effect of MECI on lipid peroxidation in STZ induced diabetic neuropathic activity.

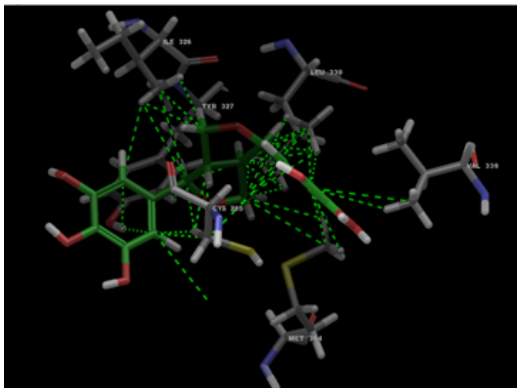
Treatment	MDA levels absorbance
Normal Control	0.749±0.0381
Negative control (STZ 55 mg/kg)	2.6318± 0.0253*, a
MECI (200 mg/kg)	1.369±0.0091*, A, a
MECI (400 mg/kg)	1.225±0.0245*, A, a
Gabapentin (3.6 mg/kg)	0.9625±0.0063*,A

Values are expressed as Mean±SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were compared with normal control group (*=p<0.01), diabetic control (A= p<0.01) and standard (a = p< 0.01), ns- non significant.

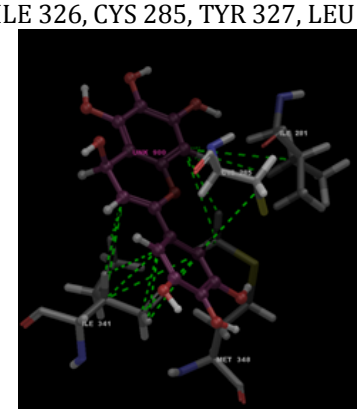
Nerve MDA level was increased significantly in diabetic rats when compared to normal control rats. The results show significant decrease in lipid peroxidation levels in treatment group rats when compared to diabetic rats. The possible mechanism by which methanolic extract of *Chrysanthemum indicum* decreased lipid peroxidation levels might be due to the presence of phenolics and flavonoids [10].

Standard precision and extra precision docking

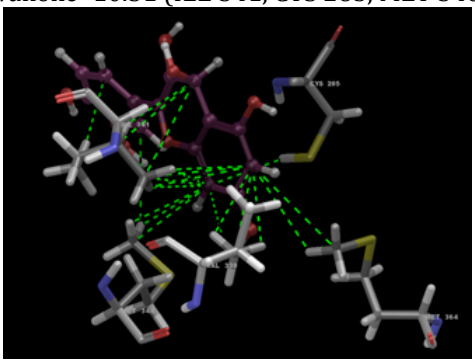
Docking was performed with structures of PKC inhibitor (PDB IDs: 2I0E), AR inhibitor (PDB IDs:4GCA), PPAR-γ agonist, (PDB IDs:1FM6). Initially, SP docking method was employed and the highest scoring compounds were subjected to XP docking. After XP analysis, the best interacting compounds were ranked based on GlideScore and the best pose of the ligand was chosen. The top ranked compounds that docked to all receptors are 5,6,7 trihydroxy flavanone, syringaresinol, tricin and quercetin. For comparison, SP GlideScore for these compounds is provided in table 5 and the compounds with high glide score for PDB ID: 1FM6 are shown in figure 1.Out of the 10 natural compounds selected initially, threecompounds like syringaresinol, 5,6,7 trihydroxy flavanone and tricin showed highest GlideScores andbinding energies [11].



Syringaresinol -14.41(ILE 326, CYS 285, TYR 327, LEU 330, MET 364, VAL 339)



5,6,7-trihydroxy flavanone -10.51 (ILE 341, CYS 285, MET 348, LEU 255, ILE 281)



Quercetin –8.34 (MET 348, ILE 341, CYS 285, VAL 339, MET 364)

Figure 3: Binding interactions of ligands with PDB ID: 1FM6

Table5: Glide scores of isolated compounds from MECI

S. No	Name of the Compound	G-Score		
		PDB ID: 4GCA	PDB ID: 1FM6	PDB ID: 2I0E
1	Syringaresinol	-13.01	-14.14	-10.12
2	5,6,7 trihydroxy flavanone	-10.84	-10.5	-11.29
3	Quercetin	-12.19	-8.34	-9.10
4	Acacetin	-10.64	-7.45	-7.76
5	Tricin	-12.12	-8.64	-6.15
6	2,4-dihydrochalcone	-10.87	-8.09	-6.31
7	Isorhamnetin	-10.67	-7.74	-8.10
8	7-hydroxy flavonone	-10.92	-7.46	-6.72
9	Liriodendrin	-	-	-8.10
10	Genkwanin	-10.21	-7.98	-9.23

DISCUSSION

Diabetic neuropathic pain which is a major complication of diabetes is considered to be relatively refractory to most of the analgesics. Reduction in MNCV (Motor Nerve Conduction Velocity) and NBF (Nerve Blood Flow) is an important feature of neuropathy. Reduced NBF leads to decreased nutritional support to nerves and failure of ATP sensitive ion exchange pumps. Demyelination, nerve ischemia and endothelial dysfunction seen in diabetes may lead to nerve conduction and blood flow deficits in experimental diabetic neuropathy[12].

Flavonoids like quercetin, acacetin, tricin, apigenin, 7-hydroxy flavanone, isorhamnetin, 5-hydroxy, 4-7-dimethoxy flavanol, 5,6,7 trihydoxy 3,4,5 trimethoxyflanon, liganans like syringaresinol and liriodendrin and glucoside like 2,4-dihydrochalcone are majorly present in the extract as reported by [13] and are used in the treatment of diabetic complications [10].

Supplementation of quercetin on the myenteric neurons and glia in the cecum of diabetic rats, have shown neuroprotective effect [14]. Quercetin has also been demonstrated to protect rat cultured dorsal root ganglion neurons against high glucose-induced injury ***in vitro*** through Nrf-2/HO-1 activation and nuclear factor K beta (NF-κB) inhibition, which may prove beneficial for the treatment of diabetic neuropathy [15]. Naringenin and apigenin neutralises oxidative stress and nerve growth factor discrepancy in experimental diabetic neuropathy. 5,6,7-trihydroxyflavone (Baicalein) alleviates diabetic peripheral neuropathy through inhibition of oxidative–nitrosative stress and p38 MAPK activation. In particular, the flavonoid baicalein alleviates motor and sensory nerve conduction velocity deficits, thermal hypoalgesia, and tactile allodynia characteristic for diabetic peripheral neuropathy, without slowing down diabetes-associated loss of intraepidermal nerve fibers and promoting their regeneration. Flavonoids like isorhamnetin increased the expression of NGF, which was achieved through ER-mediated signaling pathway [16].

Flavonoids preserve normal morphology of nervous tissues (L4 to L5 spinal cord segments, L5 DRG, and sciatic nerves), alleviating hyperglycemia, and reversing Ca^{2+} overload by increasing Ca^{2+} -ATPase activity in sciatic nerves [17].MECI extract significantly improved these functional deficits. It was seen that MECI extract more significantly decreased level of nerve MDA as compared to diabetic control rats.

If one can employ the modern computational chemistry tools for exploring the potential of the traditional medicinal system, then astonishing results can be received. In our work, structure based drug designing was used namely, molecular docking for measuring the potential of antidiabetic components and their mechanism of activity. Three receptors for diabetes like Protein Kinase-C, aldose reductase and PPAR-γ were selected in order to identify the major pathway through which MECI exhibits its antidiabetic potential.

The docking results revealed that it was PPAR-γ on which active constituents like 5,6,7 trihydroxy flavanone, syringaresinol, tricin and quercetin from MECI were found to have the highest affinity with a glide score of (-10.51, -14.14, -8.64, -8.34) for PPAR-γ. These compounds have also shown affinity for protein kinase and aldose reductase and protein kinase receptors. The role of PPAR γ in diabetes has been corroborated by many researchers.PPAR gamma (PPARγ) agonists (e.g. pioglitazone and rosiglitazone), are widely recommended for the treatment of insulin resistance-hyperglycemia [18]and to attenuate spinal nociceptive neuron activation in type II diabetic rats [19]. The recent clinical application of PPARs agonist provides a promising future to evaluate their potential as novel analgesics in the treatment of different chronic pain conditions such as diabetic neuropathy. High blood glucose level leads to the activation of AR that produces sorbitol from glucose. This reaction consumes nicotinic acid adenine dinucleotide phosphate (NADPH) and produces $NADP^+$. High consumption of NADPH reduces the level of reduced glutathione (GSH) and increases the level of oxidized glutathione (GSSG). Nevertheless, due to

the inability of sorbitol to cross the cell membrane, the accumulated sorbitol elevates the blood osmolality resulting in the loss of electrolytes [20]. High osmosis damages the cells surrounding peripheral neurons (Schwann cells) and causes the schwannopathy-related phenotype of DNP [21].
The other key enzyme sorbitol dehydrogenase converts the accumulated sorbitol to fructose via oxidation and producing nicotinic acid adenine dinucleotide (NADH). However, the increase in both sorbitol and fructose leads to some deleterious effects on nerve cells due to several reasons, including the decrease in concentration of osmolality regulator (taurine or 2-aminoethanesulfonic acid, a bile component), and insulin sensitivity regulator (myoinositol), inhibition of Na^+/K^+ ATPase pump, accumulation of intracellular Na^+, ionic homoeostasis via diminution of Protein kinase C (PKC) activity which cause swelling of axon and axon-glia dysfunction, and reduction of nerve conduction velocity (NCV)[22].
Syringaresinol (SGRS) has shown a significant potential to act as a neuromodulating agent by suppressing synaptic transmission *via* presynaptic transmitter release modulation [23]. Also, a furofuran-like lignan, syringaresinol-4-O-β-d-glucoside showed a potential efficacy in the treatment of lipid and glucose-based metabolic disorders[24]. Even Syringaresinol showed high affinity towards the receptors like 1FM6, 2I0E, 4GCA. From the above it is clear that syringaresinol which is a major component in MECI might be responsible in reliving the neuropathic pain which is a complication of diabetes.

CONCLUSION

Our studies may lay the base of further exploration of the *chrysanthemum indicum* for its antidiabetic potential and in the treatment of Diabetic neuropathy which is a major complication of Diabetes mellitus. The above findings also validate the ethnopharmacological knowledge on this plant. Hence, it can be concluded that *chrysanthemum indicum*has high potential as antidiabetic especially against PPAR-γ pathway in diabetes. However, studies are required in diabetic human subjects to prove its clinical efficacy as anti-diabetic activity.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

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