Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.com

Document heading doi:10.12980/APJTB.4.2014C1049

In vitro and *in silico* antidiabetic activity of pyran ester derivative isolated from *Tragia cannabina*

Vaiyapuri Sivajothi[1*], Shruthi Shirur Dakappa[2]

[1]*Department of Pharmaceutical Chemistry, The Oxford College of Pharmacy, Bangalore -68, Karnataka, India*

[2]*Microbiology and Cell Biology Department, Indian Institute of Science, Bangalore-560012, Karnataka, India*

PEER REVIEW

Peer reviewer

Dr. Sujan Ganapathy, P.S., Research Associate, Centre for Advanced Studies in Biosciences, Jain University, Bangalore-19, India.
Tel: 08762424836
E-mail: sujan.ganapathy@gmail.com

Comments

Diabetes mellitus is a group of metabolic diseases in which a person has high blood sugar, either because the pancreas does not produce enough insulin, or because cells do not respond to the insulin that is produced. The plant has got good ethanobotanical history, and study attempts to validate it. This scientific study support and suggest the use of this plant in treating diabetes.
Details on Page S458

ABSTRACT

Objective: To investigate the *in vitro* antidiabetic effects of isolated 4-Oxo-4H-pyran-2,6-dicarboxylic acid bis-[6-methyl-heptyl] ester from the chloroform extract of root of *Tragia cannabina* (*T. cannabina*) and AMP kinase activation property of the isolated compound.
Methods: The roots of *T. cannabina* were collected and extracted with ethanol [95% v/v] then chromatographed over silica gel 60-120 mesh of column length 100 cm and diameter 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. Then the chloroform extract was used for isolation. *In vitro* antidiabetic activity was performed with fertile eggs of White Leghorn chicks by induction of diabetes by streptozotocin.
Results: The isolated pyran ester binds very efficiently within the active pocket of AMPK with the formation of hydrogen bond and consuming less binding energy, which is good when compared to orientation of standard drug metformin. In *in vitro* antidiabetic evaluation by streptozotocin treated chick embryo the administration of isolated compound at a doses of 0.5 mg/egg and 1 mg/egg produced a significant reduction in the blood glucose levels in a dose dependant manner (P<0.01). The blood glucose level of diabetic control was (244.20±12.64) mg/dL, whereas it was (207.40 ±2.43) mg/dL (P<0.001) for isolated compound 0.5 mg/egg and 174.800±2.410 mg/dL (P<0.001) for 1 mg/ egg of the isolated compound.
Conclusions: The significant glucose levels were reduced (P<0.01) after administration of the pyran ester isolated from *T. cannabina* to streptozotocin treated chick embryo.

KEYWORDS

AMP kinase, Chick embryo, *Tragia cannabina*, 4-Oxo-4H-pyran-2, 6-dicarboxylic acid bis-[6-methyl-heptyl] ester

1. Introduction

Tragia cannabina Linn. (*T. cannabina*) commonly known as "cherukanjuru" in Tamil, India, belongs to the family Euphorbiaceae. It is a climbing shrub, found throughout the hotter parts of India. The root has been traditionally used in the treatment of diaphoresis and alternatively given in fevers to cause perspiration. A decoction of roots is useful in the treatment of bronchitis[1]. For diabetes mellitus it is one of an oriental folk medicine. This plant is used for the treatment of eczema, fevers, wheezing, including liver disorder, diabetes and viral infections[2,3]. Tragia species are rich in steroids, flavonoids, alkaloids and tannins. The literature survey revealed that the methanolic extract of *T. cannabina* posses antihyperglycemic property on streptozotocin (STZ) -induced diabetic rats[4]. The chemical examination, anticancer, antiviral and anti-inflammatory activity was screened for the *T. cannabina* extract[5-7].

*Corresponding author: Vaiyapuri Sivajothi, Department of Pharmaceutical Chemistry, The Oxford College of Pharmacy, Bangalore-68, Karnataka, India.
E-mail: writetojothi@yahoo.co.in
Tel: 080 3021 9833
Fax: 080 3021 9829
Foundation Project: Supported by dean of research/ VMCP (Grant No. 49/20011).

Article history:
Received 10 Jan 2014
Received in revised form 18 Jan, 2nd revised form 22 Jan, 3rd revised form 1 Feb 2014
Accepted 20 Feb 2014
Available online 5 April 2014

Acute and subacute toxicity of alcoholic extract of the roots of *T. cannabina* was reported[8].

Diabetes mellitus is a metabolic disorder. Chronic elevation of blood glucose level leads to retinopathy, neuropathy and peripheral vascular insufficiencies[9]. In olden days it was considered a disease of minor significance. In the 21st century, it is one of the main threads to human health. It is estimated that 300 million people will be affected by diabetes mellitus in the year 2025[10]. Increasing diabetes and obesity among the people in the world intensified search for new therapeutic treatment for diabetes mellitus[11].

The AMP-activated protein kinase (AMPK) is a highly preserved sensor of cellular energy status, found in essentially all eukaryotic genomes[12]. It is an enzyme composed of a catalytic subunit (α) and two regulatory subunits (β and γ)[13]. There are two isoforms of the catalytic subunit: AMPK α1, which is widely distributed, and AMPK α2, which is expressed in skeletal muscle, heart, and liver[14]. AMPK works as an intracellular fuel gauge that becomes activated by decreases in the ATP/ADP and phosphocreatine (PCr)/creatine ratios through mechanisms involving phosphorylation by one or more upstream AMPK kinases, allosteric activation, and a decrease in the inhibitory action of phosphatases[15]. The increase in AMPK activity stimulates the of glucose uptake in muscle, fatty acid oxidation in muscle and liver, and the inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis[16]. The AMPK system is a regulator of energy balance at both the cellular and whole-body levels, once gets activated by low energy status effects a switch from ATP-consuming anabolic pathways to ATP-producing catabolic pathways. That is why it is now appearing to be the major target to treat type 2 diabetes[17].

The objective of the present study was to investigate the *in vitro* and *in silico* antidiabetic effects of isolated 4-Oxo-4H-pyran-2,6-dicarboxylic acid bis-[6-methyl-heptyl] ester from the root of *T. cannabina* and AMP kinase activation property of the isolated compound.

2. Materials and methods

2.1. Plant material and preparation of plant extract

The roots of *T. cannabina* were collected from the local area of Salem, India in the month of October 2012 and were authenticated by the Botanist, Botanical Survey of India, Coimbatore, India. A voucher specimen (ET-30) has been stored and maintained in our laboratory. The roots were shade-dried and powdered. The powder was extracted with ethanol (95% vol/vol) using a Soxhlet apparatus. The extract was dried under reduced pressure and stored in a desiccator. The yield of extract was 3.5% (wt/wt) thus obtained crude extract was used for phytochemical screening. The ethanol extract was chromatographed over silica gel 60-120 mesh of column length 100 cm and diameter 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. Then the chloroform extract was used for isolation.

2.2. Phytochemical screening

The crude extract was qualitatively examined for the presence of various phytochemical constituents using standard tests described by Harborne[18].

2.3. Isolation of 4-Oxo-4H-pyran-2, 6-dicarboxylic acid bis-[6-methyl-heptyl] ester

The chloroform extract (5.5 g) was chromatographed over silica gel 60-120 mesh of column length 100 cm and diameter 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 15 ml portions and monitored on TLC. The fractions that showed similar spots were combined. The fractions 133-146 eluted in ethyl acetate: chloroform (10:90) solvent system, yielded a yellow coloured residue and the TLC profile showed three spots, with one major and two minor spots and rechromatographed for further purification. The yellow residue (0.150 g) obtained in this fraction was further chromatographed over silica gel 100-200 mesh of column length 75 cm and diameter 1.2 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 15 ml portions and monitored on TLC. The fractions 106-135 eluted in ethyl acetate: chloroform (10:90) solvent system, showed one major and one minor spots on TLC. It was further purified by PTLC in ethyl acetate: chloroform (20:80) solvent system, a band showing Rf value 0.544 and recrystallised in absolute alcohol to yield a yellow semi solid designated as isolated compound and the yield was 4.5 mg. This compound was subjected to physical and spectral studies for confirming the purity and characterization. The remaining fractions were not worked out because of lesser yields.

2.4. Effect of on STZ treated chick embryo

STZ was first reported to have a specific diabetogenic effect[19]. In an attempt to reduce the number of mammals used in drug research, the use of chick embryos found superior for predicting the effects of drugs[20-22]. Pancreas of chick embryo lies in duodenum that consists of a long U-shaped loop. It begins to develop rapidly during the 5th day of incubation and to acquire its definitive structure by 12th day of incubation[23]. β cells secrete insulin from the 4th or 5th day of incubation. The level of serum insulin in chick embryos increases gradually after the 12th day of incubation. The mechanism of induction of diabetes by STZ was not known.

2.5. Selection of animals

Fertile eggs of White Leghorn chicks were obtained from poultry, incubated at (37.5±0.2) °C at a relative humidity of about 65%, turned automatically every hour.

2.6. Experimental induction of diabetes

Fertile eggs of White Leghorn chicks obtained from poultry

were incubated at (37.5±0.2) °C at a relative humidity of about 65%, turned automatically every hour. For induction of diabetes, STZ was dissolved in physiological saline and sterilized through a membrane filter. A total of 300 μg/egg of STZ was injected into the albumen of fertile eggs on the 14th day of incubation.

2.7. Sample collection

Large vitelline veins of eggs were selected and marked by pencil under the florescent lamb. Eggshell of a marked range of 10×5 mm was removed by electrical drill and a drop of water was filled to project clearly an artery. Whole blood was collected from a vein of egg by means of a tuberculin syringe with a 0.55×32 mm needle. The collected blood samples were immediately centrifuged at 2000 r/min for 15 min. The serum separated out was collected in fresh serum tubes and stored in refrigerator (2-4) °C after tightly capped until analysis.

2.8. Experimental design

After the induction of diabetes the eggs were divided into seven groups of ten each as detailed bellow. Group I, Control eggs received the vehicle (saline injected into the albumen on 17th day); Group II, Diabetic control received the vehicle (saline injected into the albumen on 17th day); Group III, Diabetic control received isolated compound (0.5 mg/egg injected into the albumen on 17th day); Group IV, Diabetic control received isolated Compound (1 mg/egg injected into the albumen on 17th day); Group V, Diabetic control received insulin (4 U/egg injected into the albumen on 17th day).

The blood sample was collected on the 17th day of incubation before and after 5 min of injection of the compound and insulin. Then blood glucose was analyzed using strip method.

2.9. In silico docking studies

AutoDock tools was utilized to generate grids, calculate dock score and evaluate the conformers of inhibitors bound in the active site of AMPK as targets for antidiabetic activity. Automated docking is a graphical user interface. AutoDock 3.0 was employed to get docking and binding scores; which is implemented by Lamarckian genetic algorithm method. The ligand molecules Pyran ester and Metformin were designed and the structure was analyzed using ChemDraw Ultra 6.0. The PRODRG server was used to minimise energy of drug compounds and 3D coordinates were prepared. The protein structure file (PDB ID: 1ZON) was taken from PDB and was edited by removing the hetero atoms and C terminal oxygen was added using SwissPDB viewer. The grid map was centred at particular residues of the protein and was generated with AutoGrid. As per genetic algorithm all the torsions were allowed to rotate during docking. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters[24-27].

2.10. Statistical analysis

All data were expressed as the means±standard deviation (SD). Student's *t*-test was used to arrive at the statistically significant changes associated with various treatments. $P<0.05$ was regarded as significant.

3. Results

The chloroform extract was extracted from the root of *T. cannabina* and the preliminary phytochemical screening of crude extract indicates the presence of flavonoids, glycosides, triterpenoids, saponins and tannins. The crude extract was subjected to column chromatography to get 4-Oxo-4H-pyran-2, 6-dicarboxylic acid bis-(6-methyl-heptyl) ester (Figure 1). The IR (KBr) spectrum showed absorptions for three carbonyl groups (1 739, 1 700 and 1 696 cm^{-1}), unsaturation (1 650 cm^{-1}), isopropyl unit (1 380 and 1 461 cm^{-1}) and ether group (1 171 cm^{-1}). 1H-NMR (DMSO) spectrum exhibits two sets of doublet signals at δ 0.85 and 0.91 integrating for six protons each for isopropyl methyl groups. A 18 proton broad multiplet signals from δ 1.28 to 2.38 region were due to eight methylene and two methane protons present in identical environment showing the presence of long aliphatic side chain. It displayed signals at δ 5.40 integrated for two protons indicating the presence of two unsaturated protons it also displayed two multiplet signals at δ 4.00 and 4.63 each for two protons for two oxy methylene groups. 13C NMR exhibited three carbonyl groups at δ 227.70 a keto group and at δ 178.54 and 177.37 for two ester carbonyl groups. The signals at δ 148.50, 148.19, 107.79 and 107.51 were assigned to two carbon-carbon double bonds. The carbon of the oxy methylene group resonated at δ 58.12 and the remaining carbon chain of the long chain hydrocarbon moiety resonated from δ 49.09 to δ 12.25. Absence of any signal beyond δ 5.5 in H1-NMR and appearance of only four peaks in the unsaturated region of ^{13}C-NMR spectrum indicates the absence of benzene ring system in compound. In MASS spectrum, the molecular ion peak was observed at *m/z* 408 which indicated that the molecular weight of the compound. The melting point of the compound was recorded on electro thermal melting point apparatus and observed melting point as 168 °C.

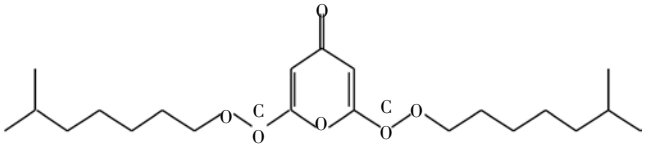

Figure 1. 4-Oxo-4H-pyran-2,6-dicarboxylic acid bis-[6-methyl-heptyl] ester.

The blood glucose level of diabetic control was (244.20±12.64) mg/dL, whereas it was (214.400±2.243) mg/dL ($P<0.001$) for compound 0.5 mg/egg and (186.200±2.631) mg/dL ($P<0.001$) for 1 mg/egg of the compound. The significant glucose levels

Table 2

Molecular docking results of AMPK.

Compounds	Binding energy	Docking energy	Inhibitory constant	H-bonds	Bond formation
PE	-3.50	-6.68	0.0	1	1A::DRG1:OAH::AMPK:C:GLN109:HN
MF	-5.54	-5.54	8.69e-005	1	MF::DRG1:HAB::AMPK:A:THR106:OG1

PE- Pyran ester, MF-Metformin

were reduced (P<0.01) after administration of the compound (Table 1). The pyran ester binds very efficiently within the active pocket of AMPK with the formation of 1 hydrogen bond with its amino acid Gln 109 of C chain. The result obtained is comparable to orientation of standard drug (Figure 2). The binding energy required is almost similar to that of standard Metformin as shown in Table 2. Studies related to improving its structure where it requires less binding energy to dock are in progress. Hence, the above pyran ester can be considered as effective treatment against diabetes.

Table 1

Effect of isolated compound on STZ treated chick embryo.

Design of Treatment	Dose (mg/kg)	Blood glucose (mg/dL)
Control	-	156.55±2.286
Saline control	-	151.00±2.150
Diabetic control	-	244.20±2.640[b]
Compound	0.5 mg/egg	214.40±2.243[a]
Compound	1.0 mg/egg	186.20±2.631[a]
Insulin	4 U/egg	157.60±1.986[a]

Values represent mean±SEM (n=6), [a]P<0.001 *v.s* diabetic control, [b]P<0.01 *v.s* control, Data were analysed by one way ANOVA followed by Tukey multiple comparison analysis

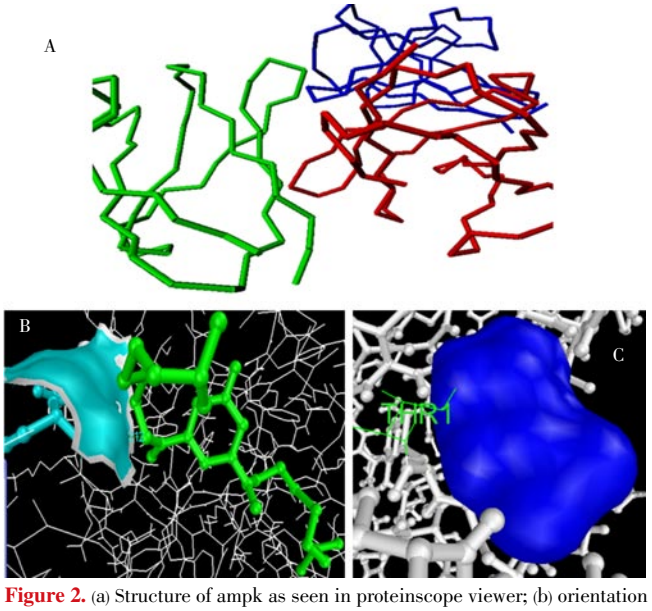

Figure 2. (a) Structure of ampk as seen in proteinscope viewer; (b) orientation of pyran ester in the active pocket of ampk; (c) enfolding of metformin in active pocket.

4. Discussion

The world's largest growing metabolic disorder is diabetes mellitus. Alternative strategies are required to manage diabetes other than modern medicine due to its enormous costs. The present manuscript discusses about the antidiabetic effects of pyran ester isolated from the chloroform extract of the root of *T. cannabina* on STZ induced egg embryo.

The chloroform extract was extracted from the root of *T. cannabina*. The preliminary phytochemical screening was done for the extract and subjected to isolation for compounds. In the present study, column chromatography and TLC eluted a compound. Based on IR, H1-NMR, 13C- NMR and MASS spectrum the compound was interpreted as 4-Oxo-4H-pyran-2,6-dicarboxylic acid bis-(6-methyl-heptyl) ester. Administration of isolated compound at a doses of 0.5 mg/egg and 1 mg/egg produced a significant reduction in the blood glucose levels in a dose dependent manner (P<0.01). The pyran ester had showed good binding efficiency in docking studies. The results obtained in *in silico* studies are in correlation with the *in vitro* studies. It has demonstrated a dose dependant efficacy. More study is required on its effects *in vivo* and to also evaluate its bio-safety and clinical potentials. However, experimental validation of the predicted compound in this direction is needed.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was financially supported by dean of research/ VMCP (Grant No. 49/20011).

Comments

Background

Diabetes mellitus is characterized by insulin resistance, which may be combined with relatively reduced insulin secretion. The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor. However, the specific defects are not known. Diabetes mellitus cases due to a known defect are classified separately. Type 2 diabetes is the most common type. Diabetes also damages the capillaries. Diabetic retinopathy, which affects blood vessel formation in the retina of the eye, can lead to visual symptoms including reduced vision and potentially blindness.

Research frontiers

The present investigate the in vitro and in silico antidiabetic effects of isolated 4-Oxo-4H-pyran-2,6-dicarboxylic acid bis-(6-methyl-heptyl) ester from the root

extract of *T. cannabina* and AMP kinase activation property of the isolated compound.

Related reports

The study validates ethanobotanical claim of plant. Standard experimental methods have been employed for both *in vitro* and in silico studies.

Innovations and breakthroughs

Plant is used as an oriental folk medicine in diabetes mellitus. The increase in AMPK activity stimulates the of glucose uptake in muscle, fatty acid oxidation in muscle and liver, and the inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis. The study orients towards deducing the relation between above mentioned facts *in vitro* and in silico.

Applications

The scientific analysis done support and suggest the use of this plant in treating diabetes. It also proves target specific drug treatment through docking studies.

Peer review

Diabetes mellitus is a group of metabolic diseases in which a person has high blood sugar, either because the pancreas does not produce enough insulin, or because cells do not respond to the insulin that is produced. The plant has got good ethanobotanical history, and study attempts to validate it. This scientific study support and suggest the use of this plant in treating diabetes.

References

[1] Council of Scientific & Industrial Research. *The wealth of India. Vol. X: A dictionary of Indian raw materials and industrial products*. New Delhi: Publication and information directorate, CSIR; 1998, p. 272.

[2] Nadkarni KM. *Indian materia medica*. Bombay, India: Popular Book Depot; 1976, p. 1226.

[3] Reddy KN, Trimurthulu G, Sudhakar Reddy C. Plants used by the ethnic people of Krishna district, Andhra Pradesh. *Indian J Tradit Knowl* 2010; **9**: 313-317.

[4] Sivajothi V, Dey A, Jayakar B, Rajkapoor B. Antihyperglycemic property of *Tragia cannabina* in streptozotocin-induced diabetic rats. *J Med Food* 2007; **10**: 361-365.

[5] Balandrin MF, Kinghorn AD, Farnsworth NR. *Human medicinal agents from plants, ACS symposium series 534*. Washington, DC: NPS Pharmaceuticals, Inc.; 1993, p. 48.

[6] Kirtikar KR, Basu BD. *Indian medicinal plants*. Bahadurganj, India: Sudhindra Nath Basu, Pâninî office; 2002, p. 2190-2191.

[7] Balandrin MF, Kinghorn AD, Farnsworth NR. *Human medicinal agents from plants, ACS symposium series 534*. Washington, DC: NPS Pharmaceuticals, Inc.; 1993, p. 2.

[8] Patel RK, Patel SB, Shah JG. Evaluation of acute and subacute toxicity of alcoholic extract of the roots of *Tragia cannabina* in rats. *Int J Pharm Sci Res* 2012; **3**: 479-482.

[9] Chehade JM, Mooradian AD. A rational approach to drug therapy of type 2 diabetes mellitus. *Drugs* 2000; **60**: 95-113.

[10] Pradeepa R, Deepa R, Mohan V. Epidemiology of diabetes in India-current perspective and future projections. *J Indian Med Assoc* 2002; **100**: 144-148.

[11] Kavi Kishor PB, Sangam S, Amrutha RN, Sri Laxmi P, Naidu KR, Rao KRSS, et al. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implication in plant growth and abiotic stress tolerance. *Curr Sci* 2005; **88**: 424-438.

[12] Hardie DG. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. *Genes Dev* 2011; **25**: 1895-1908.

[13] Kemp BE, Mitchelhill KI, Stapleton D, Michell BJ, Chen ZP, Witters LA. Dealing with energy demand: the AMP-activated protein kinase. *Trends Biochem Sci* 1999; **24**: 22-25.

[14] Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, et al. Mammalian AMP-activated protein kinase subfamily. *J BiolChem* 1996; **271**: 611-614.

[15] Frøsig C, Pehmøller C, Birk JB, Richter EA, Wojtaszewski JF. Exercise-induced TBC1D1 Ser237 phosphorylation and 14-3-3 protein binding capacity in human skeletal muscle. *J Physiol* 2010; **588**: 4539-4548.

[16] Srivastava RA, Pinkosky SL, Filippov S, Hanselman JC, Cramer CT, Newton RS. AMP-activated protein kinase: an emerging drug target to regulate imbalances in lipid and carbohydrate metabolism to treat cardio-metabolic diseases. *J Lipid Res* 2012; **53**: 2490-2514.

[17] Hardie DG, Ross FA, Hawley SA. AMP-activated protein kinase: a target for drugs both ancient and modern. *Chem Biol* 2012; **19**: 1222-1236.

[18] Harborne JB. *Phytochemical methods*. London: Chapman and Hall Ltd; 1973, p. 49-188.

[19] Alenzi FQ. Effect of Nicotinamide on experimental induced diabetes. *Iran J Allergy Asthma Immunol* 2009; **8**: 11-18.

[20] Yoshiyama Y, Kanke M. Toxic interactions between fluconazole and disopyramide in chick embryos. *Biol Pharm Bull* 2005; **28**: 151-153.

[21] Datar SP, Bhonde RR. Modeling chick to assess diabetes pathogenesis and treatment. *Rev Diabet Stud* 2011; **8**: 245-253.

[22] Maheshwari P, Baburao B, Pradeep Kumar Ch, Rama Narsimha Reddy A. Antidiabetic activity of methanolic extract of *Hiptage bengalensis* leaves in alloxan induced diabetic models. *Pak J Biol Sci* 2013; **16**(17): 844-851.

[23] Binkowski TA, Naghibzadeg S, Liang J. CASTp: computed atlas of surface topography of proteins. *Nucleic Acid Res* 2003; **31**: 3352-3355.

[24] Vidya SM, Krishna V, Manjunatha BK, Rajesh KP, Bharath BR, Manjunatha H. Antibacterial and molecular docking studies of entagenic acid, a bioactive principle from seed kernel of *Entada pursaetha DC. Med Chem Res* 2012; **21**: 1016-1022.

[25] Shruthi SD, Padmalatha Rai S, Ramachandra YL. Isolation, characterization, antibacterial, antihelminthic, and *In silico* studies of polyprenol from *Kirganelia reticulata* Baill. *Med Chem Res* 2013; **22**: 2938-2945.

[26] Annapurna HV1, Apoorva B, Ravichandran N, Arun KP, Brindha P, Swaminathan S, et al. Isolation and in silico evaluation of antidiabetic molecules of *Cynodon dactylon* (L.). *J Mol Graph Modell* 2013; **39**: 87-97.

[27] Rodriguez A, Infante D. Characterization in silico of flavonoids biosynthesis in *Theobroma cacao* L. *Network Biol* 2011; **1**: 34-45.