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Screening for Natural Inhibitors in Chinese Medicinal Plants against Glycogen Synthase Kinase 3β (GSK-3β)

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ABSTRACT

Background: The aim of this study is screening Chinese medicinal plants for inhibitors of Glycogen synthase kinase-3 (GSK-3). GSK-3 is a proline/serine protein kinase ubiquitously expressed and involved in many cellular signalling pathways. GSK-3 has emerged as one of the most attractive therapeutic targets for the development of selective inhibitors as promising new drugs for numerous pathologies, including neurodegenerative diseases and type II diabetes. Thus, the use of GSK-3 inhibitors is one of the most promising therapeutic strategies for the future treatment of these potentially life threatening diseases. **Materials and Methods:** In the aim of discovery of potential inhibitors, 42 traditional Chinese medicinal plants were screened against GSK-3β which were selected based on their folklore use. The selected plant materials were extracted with ethanol and water. *In vitro* assay was carried out to evaluate the inhibition of human GSK-3β. The Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was conducted with immortalized Hepatocyte cell line (Fa2N4) to evaluate the cytotoxicity of the plant material. **Results:** Many new ethanol and aqueous extracts showed significant inhibitory activity against GSK-3β with moderate or no cytotoxicity. Water extracts of *Prunella vulgaris, Rabdosia rubescens* and *Sarcandra glabre* have exhibited highest inhibition against GSK-3β. This in turn was supported by the fact that a good correlation exists between GSK-3β inhibitory activity and antioxidant content of the extracts. **Conclusion:** Considering the potent activity of *P. vulgaris, R. rubescens* and *S. glabre,* further isolation and characterization of individual bioactive compounds is recommended for the discovery of potent natural inhibitors of GSK-3β.

Key words: Traditional chinese plants, GSK-3β inhibitors, neurodegenerative disease, type II diabetes

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INTRODUCTION

Life threatening diseases including diabetes, cancer, neurodegeneration and inflammation associated disorders have been linked to protein phosphorylation, which is controlled by approximately 520 protein kinases and 80 protein phosphatases (Meijer *et al*., 2004). Disease can develop when these protein kinases and phosphatases malfunction, so the development of inhibitors against these enzymes has received central importance (Meijer *et al*., 2004). Glycogen synthase kinase 3β (GSK-3β) is one of best studied kinase and is known to play a crucial role in several physiological processes including insulin action, transcription, cell-division cycle, responding to DNA damage, cell death, cell survival, cell differentiation, neuronal functions, circadian rhythm and others (Meijer *et al*., 2004; Martinez *et al*., 2002; Rayasam *et al*., 2009). GSK-3β is also known to be involved in other activities including cellular signalling and canonical Wnt signalling pathway, which is important in embryonic development. Abnormalities in Wnt signalling are associated with life threatening diseases including heart attack and cancer (Rayasam *et al*., 2009; Cline *et al*., 2002; Bo *et al*., 2012).

Recent studies provide evidence that the development of Alzheimer's disease (AD) is associated with interactions between neuronal proteins and GSK-3β (Eldar-Finkelman, 2002). One such neuron specific protein is Tau, a major component of neurofibrillary tangles which are neurophysiological elements of AD (Eldar-Finkelman, 2002). It is believed that hyper phosphorylation of serine and threonine residues on the Tau protein are the primary cause of the generation of AD. Recent *in vitro* and animal studies have clearly shown

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that GSK-3 is responsible for abnormal phosphorylation on Tau protein (Eldar-Finkelman, 2002). Also, it is possible that GSK-3β activity is one of the potential pathogenic mechanisms of Amyotrophic Lateral Sclerosis (ALS) (Koh *et al*., 2007; Chung *et al*., 2008). This is suggested by the increased levels of this kinase that have been detected in the spinal cords of patients with ALS and their over expression in motor neurons (Koh *et al*., 2007). The discovery of new alternatives for the treatment of ALS is a medical need since there is no current treatment for this devastating disease.

GSK-3β also has a critical role in the regulation of Glycogen Synthase (GS) (Eldar-Finkelman, 2002) which is implicated in the development of type 2 diabetes (Eldar-Finkelman, 2002). Diabetes is the most threatened metabolic disorder affecting millions of people around the world. Studies reported that diabetes is mainly associated with the oxidative stress and protein phosphorylation controlled by protein kinases (Eldar-Finkelman, 2002). Glycogen synthesis is a key metabolic pathway involved in disposing of glucose in skeletal muscle after insulin stimulation (Eldar-Finkelman, 2002). Studies show that the over expression of GSK-3β inhibits glycogen synthesis and leads to the development of type-2 diabetes (Eldar-Finkelman, 2002). Currently there are no efficient inhibitors available for GSK-3β.

Traditional Chinese Medicinal (TCM) plants have a long history of therapeutic usage worldwide in the treatment of numerous diseases, with TCM herbal preparations accounting for up to 50% of total medicinal consumption in China (Li *et al*., 2008). Building from traditional knowledge, a renaissance of drug discovery from TCM plants has rapidly increased in the last few decades (Graziose *et al*., 2010). Many secondary metabolites and phytochemicals derived from TCM plants have become the major source of pharmaceutically important molecules (Li *et al*., 2008; Graziose *et al*., 2010). Interestingly, the bioactivity of important compounds from TCM plants is highly correlated with the ethno pharmacological knowledge derived from medicinal plants.

In TCM, the disease, 'Xiao-kezheng' is closely related to diabetes with respect to the symptoms. It is mainly attributed to deficiency of *yin* (body fluids), improper diet, overstrain and excessive sexual activities (Jia *et al*., 2003a). The treatment is directed towards eliminating the heat by nourishing yin, moistening dryness and promoting fluid production. Such medical opinion plays a potential therapeutic role by promoting blood circulation and activating the vital energy circulation. Chinese doctors often put forward prescriptions with varied medical emphasis to improve the symptoms of diabetes. Existence of herbal medicine in the treatment of diabetes dates back to 206 B.C-220 A.D. Herbal formulations are used in the form of pills, powders, plasters and tinctures (Graziose *et al*., 2010). Hundreds of prescriptions from natural medicines and preparations from folk medicines are available for the treatment of diabetic symptom. Contemporary scientific investigations and clinical studies on anti-diabetic activity has provided compelling evidence for their medicinal values (Jia *et al*., 2003b).

Several herbs and herbal formulations are approved as anti-diabetic agents in China including Yi-jin, Ke-le-nin, Yu-san-xiao, Qi-zhi, Shen-qi, Jin-qi, Xiao-ke-an (Jia *et al*., 2003a). Yi-jin is a herbal formulation with ingredients from *Panax ginseng*, *Atratylodes macrocephala*, *Poria cocos* and *Opuntia dillenii* which showed significantly lower blood glucose levels in alloxan-induced diabetic mice without affecting the glucose levels in normal mice (Jia *et al*., 2003b). It is believed that its hypoglycaemic effect might be due to its ability to restore activity of pancreatic beta cells. As per the reports from (Jia *et al*., 2003a), in an evaluation carried out among 328 type II diabetic patients in a multicentre clinical trial in Northeast China, over 85.8% of the patients showed significant clinical improvement. Despite the fact that herbal remedies are potential anti- diabetic agents; lack of target specificity remained a major hurdle for the discovery of novel therapeutics. In the recent years, GSK-3β has become a major pharmaceutically important target due to its crucial role in the development of type-2 diabetes.

The aim of the current study was to screen 42 TCM plants for their inhibition effect against GSK-3β and to identify potent inhibitors. The selection of the plants studied in this research was based on their ethno-pharmacological usage as presented in Table 1 and previous scientific investigations on their pharmacological properties. The cytotoxicity of the plants was also evaluated in order to assess their toxicity. The study was carried out on both water and ethanol extracts of all the selected plants.

MATERIALS AND METHODS

Collection of medicinal plants: The dried plant material was purchased from Beijing Tong Ren Tang Chinese Herbal Medicine shop, Sydney, Australia. The scientific names and family names are given in Table 1. The plant materials were ground to a fine powder in a grinder before extraction.

Preparation of the water extract: Approximately 3 g of each grounded plant material was taken and extracted with hot water at 121°C for 1 h. The extracted samples were centrifuged at 10,447×g for 20 min, the supernatant was transferred into a 50 mL volumetric flask adjusted the volume to 50 mL.

Table 1: Ethnobotanical use of selected Chinese medicinal plants in relation to inhibition activity against GSK-3β

S. No.	Plants names	Family	GSK/neuro protective	Reference
P1	*Acanthopanax sentico*	Araliaceae	Neuroprotective epilepsy therapy	Sucher (2006)
P2	*Actinidia arguta* Flarich.ex Miq.	Actinidiaceae	Neuroprotective activities against glutamate-induced neurotoxicity	Cho *et al.* (2012)
P3	*Akebia quinata* (Houtt.)Decne.	Lardizabalaceae	Neuroprotective activities against glutamate-induced neurotoxicity	Won and Ma (2009)
P4	*Andrographis paniculata* Wall. ex Nees	Acanthaceae	Cerebral ischemia	Chan *et al.* (2010)
P5	*Artemisia vulgaris* L.	Asteraceae	NA	
P6	*Corydalis yanhusuo* W.	Papaveraceae	NA	
P7	*Cyperus rotundus* L.	Cyperaceae	NA	
P8	*Ducheshea indica* (Andr.) Focke.	Rosaceae	NA	
P9	*Eucommia ulmoides* Oliv.	Eucommiaceae	Neuroprotective epilepsy therapy	Sucher (2006)
P10	*Hedyotis diffusa* Willd.	Rubiaceae	Neuroprotective activity	Kim *et al.* (2001)
P11	*Leonurus japonicus* Houtt.	Labiatae	NA	
P12	*Ligustrum lucidum* Ait.	Moraceae	NA	
P13	*Lysinachia christinae* Hance.	Lysimachia	NA	
P14	*Mahonia fortunei* (Lindl.)Fedde	Berberidaceae	NA	
P15	*Paeonia lactiflora* Pall.	Paeoniaceae	Neuroprotective epilepsy therapy	Sucher (2006)
P16	*Paeonis suffuticosa* Sndr.	Ranunculaceae	NA	
P17	*Plantago asiatica* L.	Plantaginaceae	Senile dementia	Zeng *et al.* (2010)
P18	*Pleione bulbocadioides*	Orchidaceae		
P19	*Pogostemon cablin* Benth.	Asteraceae		
P20	*Polygonum aviculare* L.	Polygonaceae	Neuroprotective activities against glutamate-induced neurotoxicity	
P21	*Polygonum cuspidatum* Houtt.	Polygonacae		
P22	*Poria cocos* (Schw.) Wolf	Polyporaceae	Neuroprotective epilepsy therapy	Sucher (2006)
P23	*Prunella vulgaris* L.	Lamiaceae	NA	
P24	*Pseudostellaria heterophylla*	Caryophyllaceae	NA	
P25	*Rabdosia rubescens*	Labiatae	NA	
P26	*Rheum officinale* L.	Polygonaceae	Neuroprotective effects against glutamate/NMDA (Glu/NMDA) stimulation	Lee *et al.* (2005)
P27	*Alpinae officinarum*	Zingaberaceae	NA	
P28	*Salvia miltiorrhiza* Bunge.	Caspase	Neuroprotective epilepsy therapy	Sucher (2006)
P29	*Sanguisorba officinalis* L.	Rosaceae	Oxidative stress-induced brain damage	Nguyen *et al.* (2008)
P30	*Saposhnikovia divaricata*	Apiaceae	NA	
P31	*Sarcandra glabre* (Thunb.) Nakai	Chloranthaceae	NA	
P32	*Schizandra chinensis* (Turcz.) Baill.	Schisandraceae	Senile dementia	Zeng *et al.* (2010)
P33	*Scutellaria baicalensis* Georgi.	Labiatae	Neuroprotective epilepsy therapy	Sucher (2006)
P34	*Scutellaria barbata* Don.	Labiatae	Acute ischemic stroke	Wu and Chen (2009)
P35	*Smilax glabra* Roxb_D	Smilacaceae	NA	
P36	*Solanum nigrum* L.	Solanaceae	NA	
P37	*Sophora japonica* (L.) Schott.	Fabaceae	On cerebral infaction	Chen and Hsieh (2010)
P38	*Spatholobus suberectus* Dunn.	Leguminosae	NA	
P39	*Taxillus chinensis* (DC.) Danser	Loranthaceae	Neuroprotective epilepsy therapy	Sucher (2006)
P40	*Tussilago farfara* L.	Asteraceae	Alpha-glucosidase inhibitory effect	Tchinda *et al.* (2008)
P41	*Uncaria Rhyncophylla*	Rubiaceae	Neuroprotective epilepsy therapy	Sucher (2006)
P42	*Viscum coloratum* (Komar.) Nakai	Viscaceae		

Preparation of the ethanol extract : Ground samples (3 g) were extracted with 95% ethanol on a hot water bath set at 70°C for 6 h. The extracted samples were centrifuged and the supernatant was transferred into a 50 mL volumetric flask, then the volume was adjusted to 50 mL with 95% ethanol. The samples were stored at-4°C until analysis. All water and ethanol extracts were filtered before analysis.

GSK-3β inhibition assay: Based on Kinase-Glo system and his capacity for detect the ATP present after the enzyme reaction, an *in vitro* assay was developed to evaluate the inhibition of human GSK-3β. The kinase reaction consumes ATP, but the unused ATP is used by the Luciferase for catalyzed the reaction of transformation of Bettle-Luciferin in Oxyluciferin and light. This light could be measured. The enzyme is unable to react in presence of an inhibitor and the light generated by the Luciferase is increases due to unconsumed ATP (Rinnab *et al*., 2008).

Human recombinant GSK-3β was purchased from Millipore (Millipore Iberica S.A.U.) The pre-phosphorylated polypeptide substrate GS-1 (RRRPASVPPSPSLSRHS (pS)HQRR) was synthesized by American Peptide Company (Sunnyvale, CA). Kinase-Glo Luminescent Kinase Assay was obtained from Promega (Promega Biotech Ibérica, SL). ATP and all other reagents were from Sigma-Aldrich (St. Louis, MO). The Assay buffer contained 50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA and 15 mM magnesium acetate. Enzyme Buffer contained the same formulation as assay buffer in addition to TWEEN 20 0.001% which was included to increase the stability of the enzyme.

The assays were performed in black 384-well plates. First 10 μL of test extract and 10 μL of enzyme (10 ng) dissolved in Enzyme Buffer were added to each well and mixed. After 5 min 10 μL of substrate solution was added, containing 25 μM of GS-1 substrate and 1 μM ATP. After 30 min incubation at 30°C, 30 μL of Kinase-Glo Luminescent reagent was added to each well and the luminescence was recorded using a Victor2™ Wallac multimode reader.

The inhibition percentage of GSK-3 β was determined by the equation:

$$\text{Inhibition percentage} = \left(\frac{(\text{RLU\_Sample} - \text{RLU\_Pos Contr})}{\text{RLU\_Neg Contr} - \text{RLU\_Pos Contr}}\right) \times 100$$

- RLU_Neg contr indicates the Relatives Luminiscents Units recorded for negative control
- RLU_Pos contr indicates the Relatives Luminiscents Units recorded for positive control

Assay buffer with 1% DMSO was used as negative control and Alsterpaullone 500 nM was used as positive control. Alsterpaullone and thiadiazolidinones (TDZD), both known inhibitors of the enzyme, were used as internal controls.

Determination of cytotoxic properties

Cell lines: Fa2N4, an immortalized Hepatocyte cell line, were maintained in MFE Essential Support Medium F with MFE Culture Medium Supplement A. Collagen I flasks and plates were used to grow Fa2N4 cell line. All cell cultures were kept at 37°C under a humidified atmosphere of 5% CO_2.

Cytotoxicity assay: The Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay test was applied to five cell lines for evaluation of the cytotoxic activity. It is based on the ability of drug-treated cells to reduce the yellow water soluble substrate MTT into a dark blue formazan product that is insoluble in water. Nicotinamide adenine dinucleotide (NADH) is provided directly by the cells which in turn require proper metabolic function. Therefore, the MTT reduction rate is an indicator of the functional integrity of the mitochondria and, hence, of cellular viability. For the in vitro cytotoxic activity assay, the numbers of cells per culture wells were 100,000 on 96-well plates. Samples were incubated with 60-80% of confluent culture of each cell line for 24 hours in an atmosphere of 5% CO_2 at 37°C. Each extract was tested in two different concentrations. Absorption (OD) at 570 nm was measured in a Victor2™ Wallac spectrofluorometer.

The inhibition percentage against the tested cell lines was determined by the equation:

$$\text{Inhibition percentage} = \left(\frac{1 - (\text{OD\_Sample} - \text{OD\_Pos Contr})}{\text{OD\_Neg Contr} - \text{OD\_Pos Contr}}\right) \times 100$$

- OD_Neg Contr indicates the measured optical density for negative control at 570 nm
- OD_Pos Contr indicates the measured optical density for positive control at 570 nm

Medium with 1% DMSO was used as negative control and medium with 2 mM of Methyl Methane Sulfonate (MMS) was used as positive control. Actinomycin D (250, 50, 12.5 μM), doxorubicin A (250, 50, 12.5 μM) and rotenone (250, 125 μM) were used as internal controls.

Data presentation and analysis: Data for the GSK-3 β screen and MTT cytotoxicity assay were analyzed using the gene data screener program (Genedata AG, Switzerland). DMSO at the same concentration used for the solubility of the compound, served as the negative control for assays, while 500 nM Alsterpaullone and 2 mM MMS were used as positive controls. In all results the RZ' factor (Zhang *et al*., 1999) was between 0.7-0.9. The comparative analysis of various data sets was done using statistical correlation, was represented using Spotfire Silver (Tibco).

RESULTS

Traditional Chinese Medicinal (TCM) plants have been used for centuries as dietary supplements for symptoms of diabetes and neurological diseases. However, the *in vitro* effects of these extracts have not been determined. This study reports the GSK-3β inhibitory activities of water and ethanol extracts of 42 TCM plants. The inhibitory activities are expressed in terms of IC_{50} values (μg mL^{-1}) of both aqueous and ethanol extracts. These results from all the selected plants are presented in Table 2 and Fig. 1 and 2. It is interesting to note that significant relationship was observed between the GSK-3β inhibitory properties and their traditional anti-diabetic and neuroprotective activities (Table 1). Significantly larger number of water extracts (33%, Fig. 1a) showed inhibitory activity without toxicity compared to the number of ethanol extracts (21% Fig. 2a) that showed activity.

Amongst the water extracts, 14 plants showed significant inhibitory activity (qIC_{50}<20 μg mL^{-1}) with no or minimal cytotoxicity (Table 2 and Fig. 1). Most active plants are: *P. vulgaris* (qIC_{50}<10.3 μg mL^{-1} and non cytotoxic), *R. rubescens* (qIC_{50}<2.58 μg mL^{-1} and moderately cytotoxic), *Sanguisorba officinalis* (qIC_{50}<2.58 μg mL^{-1} and cytotoxic) and *S. glabre* (qIC_{50}<2.58 μg mL^{-1} and non-cytotoxic). Several other plant extracts exhibited significant inhibitory activity

Table 2: Inhibition activities of ethanol and water extracts of selected Chinese medicinal plants against GSK-3β and their toxicities

	qIC50 (μg mL^{-1})		Max activity		Toxicity	
S. No	Water extracts	Ethanol extracts	Water extracts	Ethanol extracts	Water extracts	Ethanol extracts
P1	26.3	ND	56.7	0.0	C	
P2	15.7	88.8	68.7	99.1	MC	NC
P3	58.1	46.6	50.6	93.1	NC	C
P4	>330	202.7	30.2	66.2	NC	NC
P5	4.3	286.3	64.7	57.3	MC	C
P6	ND	210.3	0.0	58.3	NC	NC
P7	49.2	98.3	55.7	86.1	NC	MC
P8	13.1	53.4	67.6	104.9	MC	MC
P9	40.0	80.1	49.0	93.5	NC	MC
P10	53.9	75.8	44.4	87.3	NC	C
P11	13.6	43.4	62.2	95.5	C	C
P12	20.1	74.2	52.7	84.0	MC	MC
P13	ND	140.2	0.0	80.3	NC	MC
P14	>330	34.6	51.4	101.7	NC	NC
P15	309.6	64.8	49.4	96.9	NC	NC
P16	125.9	106.5	65.7	93.1	NC	NC
P17	ND	193.6	0.0	73.8	NC	MC
P18	59.4	ND	42.2	0.0	NC	
P19	>330	241.2	39.0	62.6	NC	C
P20	4.0	89.0	198.9	88.8	NC	NC
P21	65.3	166.8	58.6	73.4	NC	NC
P22	ND	141.1	0.0	76.1	NC	NC
P23	<10.3	28.3	64.8	88.9	NC	C
P24	ND	>330.0	0.0	1.0	NC	NC
P25	<2.58	ND	91.0	0.0	MC	
P26	113.2	253.8	68.9	65.0	NC	MC
P27	7.2	93.7	73.2	88.3	NC	NC
P28	102.0	193.9	74.8	80.0	NC	MC
P29	<2.58	<2.58	80.3	93.6	C	NC
P30	>330	>330.0	-7.0	-7.2	NC	NC
P31	<2.58	87.0	68.8	125.6	NC	MC
P32	>330	ND	48.2	0.0	NC	
P33	20.4	57.5	77.7	92.9	NC	MC
P34	20.0	48.1	72.7	84.3	MC	MC
P35	>330	>330.0	50.7	-1.5	NC	NC
P36	>330	224.3	12.5	69.2	NC	C
P37	34.5	187.8	66.9	70.4	NC	NC
P38	21.7	128.8	99.6	96.7	NC	NC
P39	9.2	77.2	66.1	104.2	NC	NC
P40	28.7	83.5	66.2	94.5	NC	C
P41	34.9	>330.0	71.8	-4.8	NC	NC
P42	ND	24.7	0.0	98.3	NC	C

ND: No data, C: Cytotoxic, MC: Moderate cytotoxic, NC: Non-cytotoxic

(Table 2 and Fig. 1) indicating their high therapeutic index. However, it should be noted that some of the water extracts (25%, Fig. 1b) were active but toxic and several other plant extracts (42%, Fig. 1c) did not show any activity.

In the case of ethanol extracts, only *S. officinalis* (qIC_{50}<2.58 μg mL^{-1} and non-cytotoxic) showed significant inhibitory activity. Approximately, one third of the ethanol extracts (34%, Fig. 2b) showed inhibitory activity with toxicity. It is interesting to note that the inhibitory activities and toxicities are significantly different for water and ethanol extracts (Table 2, Fig. 1 and 2).

In order to further understand the GSK-3β inhibitory activities of the selected medicinal herbs in terms of their antioxidant contents (total phenolics and flavonoids), 11 water extracts and 10 ethanol extracts were selected (Table 3 and 4) and correlation plots were developed (Fig. 3 and 4). Amongst the water extracts the GSK-3β inhibitory activity showed significant correlation with total phenolics content (Fig. 3a, R^2 = 0.5146, $p<0.05$) and also with the total flavonoid content (Fig. 3b, R^2 = 0.5529, $p<0.05$). The correlation of GSK-3β inhibitory activities to their total phenolics content (Fig. 4a, R^2 = 0.7651, $p<0.05$) and flavonoid content (Fig. 4b, R^2 = 0.5384, $p<0.05$) was also significant in ethanol extracts.

The results presented in this study are in agreement with the fact that the total phenolics and flavonoid contents are contributors to the GSK-3β inhibitory

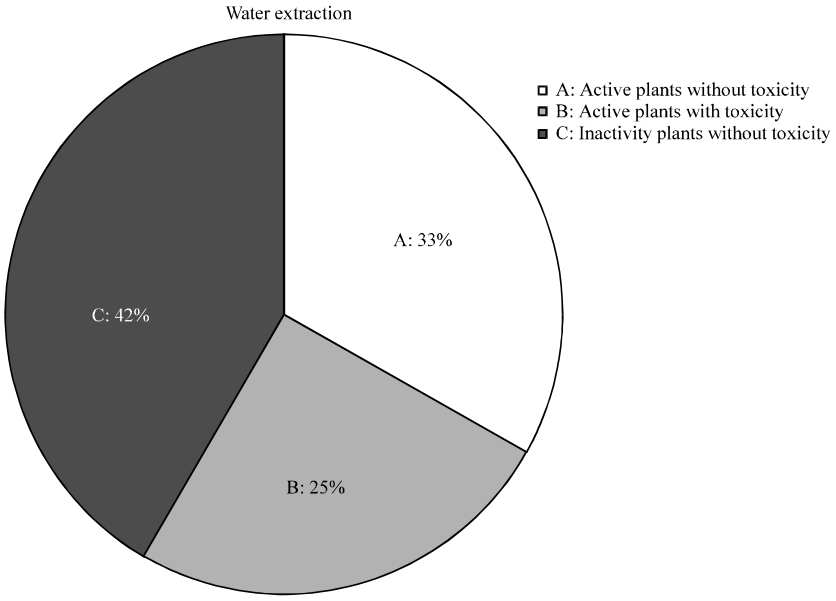

Fig. 1: Diagrammatic visualization schemes for the correlation of inhibition activity against GSK-3β from selected Chinese medicinal plant water extracts and their toxicity, (a) Plants with inhibit activity and without toxicity: P1, P3, P4, P10, P11, P13, P14, P15, P16, P17, P26 and P29, (b) Plants with inhibit activity and with toxicity: P2, P5, P6, P7, P8, P9, P12, P27 and P28, (c) Plants without inhibit activity and also without toxicity: P18, P19, P20, P21, P22, P23, P24, P25, P30, P31, P32, P33, P34, P35and P36

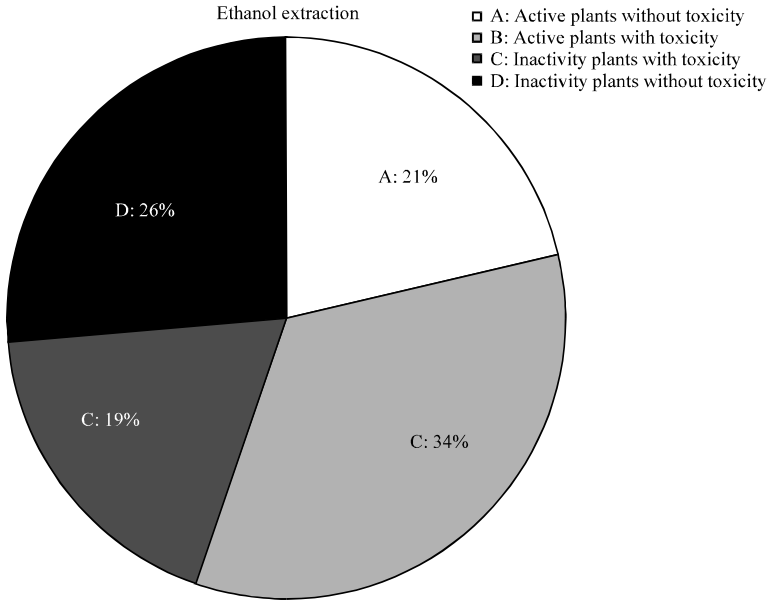

Fig. 2: Diagrammatic visualization schemes for the correlation of Inhibition activity against GSK-3β from selected Chinese medicinal plant ethanol extracts and their toxicity, (a) Plants with inhibit activity and without toxicity: P3, P9, P12, P16, P17, P18, P20 and P34, (b) Plants with inhibit activity and with toxicity: P1, P2, P4, P5, P6, P7, P8, P10, P11, P13, P14, P15 and P19, (c) Plants without inhibit activity and with toxicity: P21, P23, P24, P25, P28, P29, P35, P36, P37 and P38, (d) Plants without inhibit activity and also without toxicity: P22, P26, P27, P30, P31, P32 and P33

Table 3: Water extracts of herbs having correlation between anti-diabetic activity and antioxidant compounds

No.	Plants
1	*Artemisia vulgaris*
2	*Ducheshea indica*
3	*Ligustrum lucidum*
4	*Scutellaria baicalensis* Georgi.
5	*Polygonum cuspidatum* Sieb.et Zucc
6	*Rheum officinale* Bail
7	*Paeonia suffuticosa*
8	*Alpinae officinarum*
9	*Paeonia lactiflora*
10	*Schizandra chinensis*
11	*Solanum nigrum*

Table 4: Ethanol extracts of herbs having correlation between anti-diabetic activity and antioxidant compounds

No.	Plants
a	*Sanguisorba officinalis*
b	*Taxillus chinensis* (DC.) Danser
c	*Spatholobus suberectus*
d	*Actinidia arguta*
e	*Smilax glabra*
f	*Artemisia vulgaris*
g	*Uncaria rhyncophylla*
h	*Saposhnikovia divaricata*
i	*Pogostemon cablin*
j	*Plantago asiatica*

activity of herbal medicine. The water extracts of the herbs *A. vulgaris, D. indica, L. lucidum* and *S. baicalensis* showed high GSK-3β inhibitory activity and also have high total phenolics and flavonoid content (Table 2, 3 and Fig. 3). The ethanol extracts of the herbs *S. officinalis, T. chinensis, S. suberectus* and *A. arguta* showed high GSK-3β inhibitory activity and also have high total phenolics and flavonoid content (Table 2, 4 and Fig. 4).

DISCUSSION

Many plants, that showed significant activity with water extracts, have displayed minimal or no activity with ethanol extracts. A diagrammatic representation of the inhibitory activities of the plants and their cytotoxic properties are given in Fig. 1 (water extracts) and Fig. 2 (ethanol extracts). The results of this study clearly indicate that the w ater extracts display superior GSK-3β inhibitory activity when compared with ethanol extracts. Also the results indicate that water extracts are less toxic than the ethanol extracts and safer to use.

Following important conclusions can be drawn from Fig. 1 and 2:

- Water extracts generally showed higher activity when compared with ethanol extracts
- Number of plants that showed activity when extracted with water were larger compared to those extracted with ethanol

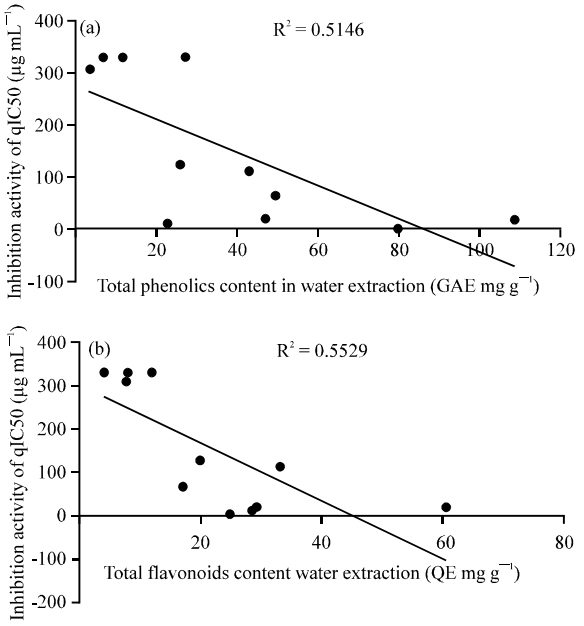

Fig. 3(a-b): Correlation between inhibition activity against GSK-3β and the total phenolics content (3A) and flavonoids content (3B) in water extracts

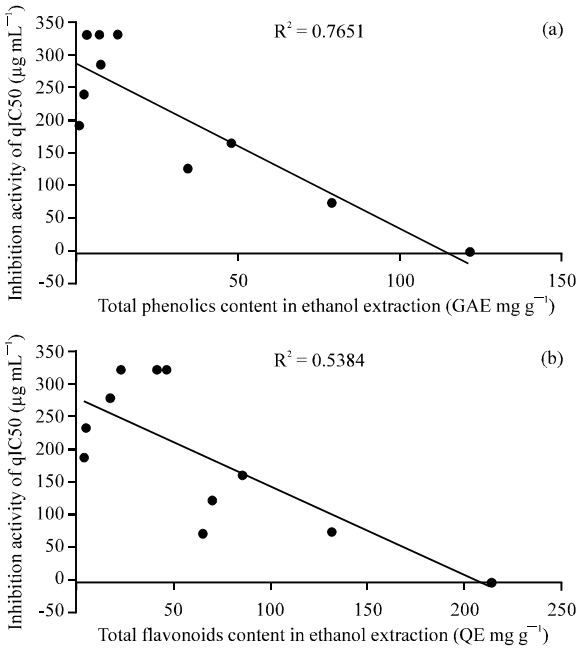

Fig. 4(a-b): Correlation between inhibition activity against GSK-3β and the total phenolics content (4A) and flavonoids content (4B) in ethanol extracts

- Number of ethanol extracts that showed toxicity were much larger than those of water extracts

- Hence, water extracts are more preferable and safer which is consistent with traditional practice

Modern drug discovery is often inspired from the traditional knowledge of medicinal plants. In this regard, TCM plants have received huge interest due to their long history of usage in the treatment of various disorders. Traditionally, the TCM plants were consumed primarily in the form of hot water extraction or alcohol extraction (Parekh *et al*., 2005; Ravipati *et al*., 2012). One of the critical steps in the biological screening of medicinal plants is the type of extraction used, as a matter of fact each extraction method yields different active ingredients (Parekh *et al*., 2005; Ravipati *et al*., 2012). Water and ethanol extraction methods are both cost effective, easy to prepare the plant material and they are non-toxic at minimal dosages. The current study on GSK-3β inhibition potential of plant material was therefore conducted using both water and ethanol extracts. The cytotoxic properties of all the plant extracts were also evaluated as described in a previous study (Ravipati *et al*., 2013).

It has been reported in the literature that antioxidants play a crucial role in delineating the diabetic complications (Rahimi *et al*., 2005; Tchinda *et al*., 2008). In addition, studies have also shown a positive correlation between antioxidant content and α-glucosidase inhibition suggesting the role of antioxidants in the regulation of diabetic conditions (Apostolidis and Lee, 2010). For instance boswellic acid, ellagic acid, quercetin, rutin and normoglycemic are flavonoids that showed significant hypoglycemic and anti-diabetic activity in rats with STZ-nicotinamide induced type 2 diabetes. After 14 days of administration of STZ-nicotinamide in rats, the total cholesterol, triglyceride was significantly diminished, suggesting the anti-diabetic activities of flavonoids (Apostolidis and Lee, 2010). In agreement with previous studies, the GSK-3β inhibitory activities of the selected plants observed in this study are significantly correlated with their antioxidant potential (Ravipati *et al*., 2012; Ravipati *et al*., 2013). *Tussilago farfara, Salvia miltiorrhiza* and *Paeouis suffuticosa* contain large quantities of antioxidants and trace elements with significant antioxidant and anti-inflammatory activities (Ravipati *et al*., 2012; Ravipati *et al*., 2013). It is also observed that these plants exerted their maximum inhibitory activity against GSK-3β.

It is conceivable that modern drug discovery is inspired from the ethno-pharmacological evidence. Traditionally, the extracts of TCM plants were used in the treatment of diabetes and the whole plant extract was used in the treatment of epilepsy, irritability, insomnia and anxiety disorders (Ravipati *et al*., 2012; Ravipati *et al*., 2013). Enzyme assay guided fractionation studies carried out by (Gao *et al*., 2008) revealed the significant inhibitory activity of methanol extract of *T. farfara* showed highest inhibition against maltase (Apostolidis and Lee, 2010). Further characterization of this extract revealed the presence of 3, 4-dicaffeoylquinic acid (Parekh *et al*., 2005), 3, 5-dicaffeoylquinic acid (Ravipati *et al*., 2012) and 4,5-dicaffeoylquinic acid. An investigation carried out by (Huang *et al*., 2012) showed high therapeutic potential on diabetic conditions if the plants possess high total polyphenolic content (Huang *et al*., 2012). The rat models showed a significant decrease in the blood glucose, total cholesterol, triglyceride and blood urea nitrogen and increase in insulin sensitivity index, suggesting the anti-diabetic properties of the plant extracts (Huang *et al*., 2012). Another plant *P. suffruticosa* has been used in anti-diabetic herbal formulations (Shin *et al*., 2012) in order to evaluated their anti-diabetic properties *in vitro* models. These studies showed significant anti-diabetic affect by inhibiting the uptake of glucose (Lau *et al*., 2007). Studies (Ha *et al*., 2009) also suggest that the antioxidant content of medicinal plants/herbs may play a role in anti-diabetic properties. Remarkable fact to be noted is that the studies involving *in vitro* inhibitory activities gave most valuable supporting evidence for their traditional use as anti-diabetics and for other diseases like cancer, Alzheimr's and inflammation.

CONCLUSION

Traditional Chinese Medicinal plants have been the source of many pharmaceutical compounds that are available in the market today. Current study is the first of its kind to screen large number of plant extracts for their GSK-3β inhibitory activities. Of all the plants studied, *P. vulgaris, R. rubescens* and *S. glabre,* showed highest GSK-3β inhibition. The results presented in this study clearly indicate that water is the best extraction solvent for isolating the compounds with GSK-3β inhibitory activity from medicinal herbs. Significant correlation was found between the antioxidant content and anti-diabetic ability of the plants. Further investigations employing various separation techniques on these plants could lead to the discovery of promising inhibitors of GSK-3β. Currently, bioactivity guided fractionation and characterization of novel class of molecules from these plants are underway in our laboratory.

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