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Evaluation of Misai Kucing (*Orthosiphon stamineus*) extract on diabetic cell line

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Abstract. *Orthosiphon stamineus*, locally known as Misai Kucing, is a traditional medicinal plant used to treat various ailments, including diabetes. However, limited scientific research has confirmed its anti-diabetic potential. This study aimed to evaluate the effects of *O. stamineus* extracts on diabetic cell lines. The plant was dried, ground, and extracted using four different solvents: water, ethanol, ethyl acetate, and hexane. The crude yield from these extractions was highest in water, followed by ethanol, ethyl acetate, and hexane. *In vitro* toxicity assays were conducted on the 3T3-L1 pre-adipocyte cell line to assess the viability of cells treated with water and ethanolic extracts. The extracts were tested at various concentrations to determine their toxicity. Concentrations below 1.25 x 10^{-1} mg/mL for both extracts were non-toxic, with more than 50% viable cells observed. However, at higher concentrations (1.25 x 10^{-1} mg/mL), the water extract showed toxicity. Non-toxic concentrations (ranging from 0 to 0.06 mg/mL) were used to measure glucose uptake in the 3T3-L1 diabetic cell line by incubating the cells with a fluorescent D-glucose analog, 2-NBDG. The results showed a reduction in 2-NBDG uptake in cells treated with the extracts compared to untreated cells, indicating the potential to influence glucose metabolism. This suggests that water and ethanolic extracts of *O. stamineus* could play a role in managing diabetes by improving glucose regulation, supporting its traditional use as an anti-diabetic remedy and highlighting its medicinal potential.

Keywords: *orthosiphon stamineus*, misai kucing, anti-diabetic, glucose uptake, 3T3-L1 cell line, medicinal plant extract

INTRODUCTION

Herbal medicines have gained significant attention as alternative treatments in recent years in Malaysia and are commonly sold as dietary supplements. In Malaysia, herbs are widely used by the public for medicinal purposes, food, and supplements. Various parts of the plant, such as leaves, stems, roots, flowers, and seeds, are utilized for their healing properties. A growing variety of herbal products are available in local markets, with many sold as over-the-counter (OTC) medicines. The use of herbal medicine spans across many countries, from Europe to Asia. According to the World Health Organization (WHO), 80% of the global population relies on herbal medicine. Using plants for healing is a key component in systems like Ayurveda, homeopathy, and traditional medicine. Globally, herbal medicine has evolved from simple remedies such as herbal teas or crude tablets to more refined and standardized forms in modern herbal treatments.

There are at least 250,000 species of flowering plants recorded worldwide, with 150,000 found in the tropics. Southeast Asia is home to

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approximately 35,000 species of flowering plants, of which 8,000 are found in Malaysia (Muhammad & Mustafa, 1994). Traditionally, unprocessed herbs were used for medicinal purposes, often prepared by boiling plant parts in water to make a drink. Today, herbal preparations have evolved, with herbs now extracted using different organic solvents to enhance the efficiency and quality of the preparation for therapeutic purposes. However, the public's understanding of herbal medicine remains limited, and more scientifically proven research is needed.

Consumers often prefer herbal products over modern drugs, particularly after prolonged use, due to the belief that they have fewer side effects. However, the pharmacological action of certain drugs can change or lead to unwanted side effects, such as toxicity, when combined with herbs. This highlights the importance of ensuring the efficacy, safety, and quality of herbal remedies by providing scientific evidence on potential herb-drug interactions and toxicity.

Orthosiphon stamineus, a herbaceous shrub from the Lamiaceae family, is locally known as Misai Kucing and is commonly found in Southeast Asia and tropical regions. According to Yam *et al.* (2010), *O. stamineus* possesses confirmed anti-inflammatory and non-narcotic analgesic properties, making it effective for treating pain and inflammation. In Southeast Asia, it is widely used for various health conditions (Rao *et al.*, 2014), particularly for kidney and bladder ailments, such as kidney stones and nephritis. Additionally, it has antiallergenic, anti-hypertensive, and anti-inflammatory properties, and is used to treat gout, diabetes, hypertension, and rheumatism. The plant is often included in products requiring a safe diuretic action, such as for diabetes, detoxification, water retention, hypertension, weight loss, and kidney stones.

Research into herbal medicines for treating diabetes using natural products has been increasing globally (Rao *et al.*, 2014). In Myanmar, *O. stamineus* leaves are used as an antidiabetic remedy (Han *et al.*, 2008). The plant extract helps lower blood sugar levels (hypoglycaemia) and maintains glucose concentrations near normal. Diabetes is a metabolic disorder affecting carbohydrate, fat, and protein metabolism. Intestinal glycosidase enzymes play a key role in carbohydrate digestion and absorption. Therefore, inhibiting these enzymes could delay digestion, reduce post-meal blood glucose spikes, and mimic the effects of dieting on hyperglycaemia, hyperinsulinemia, and hyper-triglyceridemia. One effective strategy to control carbohydrate-related diseases is to limit intestinal carbohydrate digestion, with α-glucosidase inhibitors being useful for managing conditions like diabetes, obesity, hyperlipidemia, and hyperlipoproteinemia (Mohamed *et al.*, 2012). In addition, hexane extracts of *O. stamineus* have been demonstrated to reduce glucotoxicity in pancreatic INS-1 cells and increase insulin mRNA expression. This effect is likely mediated via the PI3K/Akt signaling pathway, a critical regulator of cellular insulin responses. Moreover, the hexane extract enhanced insulin secretion under glucose-stimulated conditions and provided protective effects against glucotoxic stress, suggesting its potential as a therapeutic agent for managing insulin resistance and diabetes (Lee *et al.*, 2015). A more recent integrative analysis using network pharmacology have predicted potential pathways through which the plant's compounds exert glucose-lowering effects and antioxidant activities of *O. stamineus* (Wang *et al.*, 2023).

Many scientific studies have reported on *O. stamineus*, but toxicology information and safety data on this herb remain limited and insufficiently explored. Numerous herbal products can cause hepatic toxicity after prolonged use. For instance, plants like mushrooms and stone fruits have been identified as potentially toxic and even fatal in some cases (Fenton, 2002). It is crucial for herbalists and manufacturers to understand the relationship between the pharmacological activities of active herbal compounds and their possible interactions with other drugs when consumed concurrently. The IL_{50} value estimates the potency of toxicants, indicating the concentration at which 50% of cells experience lethality. The therapeutic benefits of medicinal plants often stem from their antioxidant properties, with phenolic compounds showing diverse biological activities such as anti-inflammatory, anticarcinogenic, and anti-atherosclerotic effects, often linked to their antioxidant capabilities.

The role of *O. stamineus* in managing gestational diabetes remains understudied, despite its promising anti-diabetic properties. Given the

rising prevalence of gestational diabetes, exploring the therapeutic potential of this herb could lead to more effective, natural alternatives for managing the condition, with fewer side effects than conventional treatments. In this study, we investigated the potential effects of *O. stamineus* on gestational diabetes. Although previous animal and *in vitro* studies have explored its antidiabetic effects, the mechanisms in maternal hyperglycaemia are not fully understood (Sriplang *et al.*, 2007; Mohamed *et al.*, 2012). This study evaluated *O. stamineus* extract on a 3T3-L1 diabetic cell line to determine its glucose-lowering potential, highlighting its promise as an alternative to insulin or oral hypoglycaemic agents in diabetes treatment.

MATERIALS AND METHODS

Plant material

Dried *O. stamineus* plants as seen in Figure 1 were sourced from Taman Universiti, 81300, Skudai, Johor. The dried samples were ground into a fine powder using a universal blender (Panasonic, China). The resulting powder was then stored in a dryer (Protech, United Kingdom) at 30°C for further use.

Extraction of O. stamineus

The shade-dried and powdered *O. stamineus* was extracted using four different solvents: water (H_2O), ethanol (C_2H_6O) (96% B.P. grade, Fisher Scientific Singapore), ethyl acetate ($C_4H_8O_2$) (HPLC grade, Sigma-Aldrich), and hexane (C_6H_{14}) (95%, Sigma-Aldrich). A 10 g sample of powdered *O. stamineus* was weighed using a precision balance (Kern, Germany) and extracted with 100 mL of each solvent at 40°C for 4 hours in a water bath (WiseBath, Germany) following the protocol by Akowuah *et al.* (2005). The extracts were then filtered through Whatman No. 1 filter paper using a Buchner funnel under vacuum (Eyela N-1000, Japan). The volume of the filtered extracts was measured using a measuring cylinder. The filtrates were concentrated to dryness using a rotary evaporator under vacuum (Rotary Evaporator N-1000, Eyela Oilbath OSB-2000, and Eyela N-1000 Aspirator, Japan) to obtain crude extracts. The evaporation was carried out at 40°C to minimize potential degradation of the phytochemicals in the samples. The extraction process was performed in triplicate, and the extraction yield for both water and organic solvents was calculated using the following equation from Pin *et al.* (2010):

$$Y = \left(\frac{W_d}{V_r}\right) \times R_{ss} \times 100$$

Where, W_d is the weight of dried extract (g), V_e is the volume of aqueous filtered (mL) and R_{ss} is the ratio of solvent to solid (mLg^{-1}).

Figure 1. Preparation of the dried *O. stamineus* plant sample to the powdered form before extraction process. (a) Live plants *O. stamineus*, (b) dried *O. stamineus* plants and (c) powdered form of *O. stamineus*.

The dried extracts were reconstituted in a dimethyl sulfoxide (DMSO)-ethanol mixture (60:40 v/v) to achieve a stock concentration of 2 mg/mL, as described by Chan *et al.* (2015). The stock solutions were then sterilized using a 0.45 μm syringe filter, employing a 5 cc/mL syringe and a 0.45 μm nylon syringe filter. The sterilized stock solutions were stored at -20°C until further use.

Preparation of complete growth medium

A 500 mL complete growth medium (CGM) was prepared by mixing 89% Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 10% Bovine Calf Serum (BCS), and 1% Penicillin-Streptomycin antibiotic. This complete growth medium was utilized for cell culture.

Cell thawing and reviving

Cell thawing was performed following the manufacturer's protocol, using 3T3-L1 (mouse embryonic) cell lines. The 3T3-L1 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultivated in 25-cm^2 culture flasks (T-25) with complete growth medium at 37°C in a humidified atmosphere containing 5% CO_2. The cells were sub-cultured before reaching 80%-90% confluency. For subculture, samples were frozen in a mixture of 90% complete growth medium (89% DMEM, 10% BCS, and 1% Penicillin-Streptomycin) and 10% dimethyl sulfoxide (DMSO), as described by Zebisch *et al.* (2012). To initiate thawing, 10 mL of complete growth medium (CGM) was pipetted into a T-25 flask using a sterile 10 mL serological pipette. The fresh medium in the T-25 flask was pre-warmed to 37°C. Next, 1 mL of frozen cells from a cryovial was thawed in a water bath (Branson, USA) at 37°C until the ice melted. The thawed cell suspension was then added to the pre-warmed fresh medium in the T-25 culture flask. The culture was incubated in an incubator (Nuaire, USA) at 37°C in a humidified atmosphere with 5% CO_2 for 48 hours (two days).

Cell passaging of 3T3-L1 pre-adipocyte cell lines

The subculture of 3T3-L1 cells was performed in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Calf Bovine Serum (CBS) and 1% penicillin, in a 5% CO_2 incubator at 37 °C for 48 hours. Once the cells reached approximately 70%-80% confluency in a T-25 culture flask, they were split into two T-75 culture flasks. Fresh medium was prepared in the two T-75 culture flasks and pre-warmed in the incubator before subculturing. The culture medium in the T-25 culture flask was removed and discarded. The cells were washed with 5 mL of D-PBS, which was gently added to the side of the vessel opposite the attached cell layer to avoid disturbing the cells. The wash solution (D-PBS) was discarded and removed from the culture vessel. Then, 2 mL of trypsin was added to the T-25 culture flask to detach the cells. The trypsin, a dissociation reagent, was applied sufficiently to cover the cell layer, and the container was gently rocked to ensure complete coverage. The culture vessel was incubated for approximately 5 minutes. The cells were then observed under a microscope (Carl Zeiss, Germany) for detachment. The culture vessel was gently tapped to detach any remaining cells. Next, 5 mL of complete growth medium was added to the culture vessel, and the cell suspension was transferred to a 15 mL Falcon tube. The cells were then distributed into the two pre-warmed T-75 culture flasks based on the calculated amount. The new culture vessels were incubated in the incubator for another 48 hours before the next passage.

Cryopreservation of cultured 3T3-L1 cell lines

For cryopreservation, 10 mL of freezing medium was prepared and stored at 2°C. The freezing medium for 3T3-L1 cell lines consisted of 95% complete growth medium and 5% dimethyl sulfoxide (DMSO). The cells were gently detached from the culture vessel following the sub-culture procedure described previously. After detachment, the cells were re-suspended in complete growth medium and transferred into a 15 mL Falcon tube. The cell suspension was then centrifuged using a centrifuge (Zentrifugen, Germany) at 180 RCF for 5 minutes. The supernatant was discarded carefully to avoid disturbing the cell pellet, which was then re-suspended in 1 mL of cold freezing medium. The cell suspension was dispensed into cryogenic storage vials, ensuring the cells were mixed gently and frequently to maintain a homogeneous suspension. Each vial was labelled appropriately

with the cell line name and date, and sealed securely with parafilm. The vials were initially frozen at -20°C overnight before being transferred to a -80°C freezer (Sanyo, Japan) for an additional overnight period. Finally, the frozen cells were transferred and stored in liquid nitrogen for long-term preservation.

In vitro cytotoxicity assay by MTT assay

The colorimetric MTT assay, which utilizes 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, is a common method for measuring cell viability and has been described by Vijayalakshmi and Selvaraj (2018) with some modifications. MTT reagent was prepared by dissolving it in D-PBS at a concentration of 5 mg/mL and filtering it to remove any insoluble residues. This solution was then diluted in PBS to achieve a final concentration of 0.5 mg/mL. A confluent Vero cell monolayer, at approximately 80% confluency, was seeded in 96-well sterile culture plates. Following an incubation period of 48 hours at 37°C in a 5% CO_2 incubator, 100 µL of each two-fold serially diluted extract concentration (ranging from 1 mg/mL to 3.91 x 10^{-3} mg/mL) was added to each well and incubated for an additional 48 hours. Control groups, including a medium control (blank medium/DMSO) and a cell control (cells without extract treatment), were included on the same plates. After the incubation period, cell viability was assessed using the MTT colorimetric assay as described by Mosmann (1983), with modifications. Ten microliters of MTT solution was added to each well, followed by a 4-hour incubation at 37°C in a 5% CO_2 incubator, conducted in the absence of light due to the light-sensitive nature of MTT reagent. The reaction was terminated by aspirating the MTT reagent, and the resulting formazan crystals were dissolved in 100 µL of DMSO in each well. Absorbance was measured at 570 nm using a microplate reader. The percentage of viable cells was calculated using the following formula:

$$\% \text{ of viable cell} = \frac{(\text{abs sample} - \text{abs blank})}{(\text{abs control} - \text{abs blank})} \times 100$$

Differentiation of pre-adipocyte cells to adipocyte cells

The differentiation of pre-adipocyte cells into adipocytes was conducted based on the protocol published by Son *et al.* (2011), with some modifications. Pre-adipocyte cells were maintained until they reached 80% confluency after several passages. Upon reaching confluency, the cells were incubated for an additional 48 hours before the differentiation process commenced. The medium used for 3T3-L1 pre-adipocytes was discarded and replaced with an appropriate volume of differentiation medium, consisting of DMEM with 10% Bovine Calf Serum (BCS). To induce differentiation into mature adipocytes, the cells were treated with a specific MDI (methylisobutylxanthine, dexamethasone, and insulin) differentiation stimulus. The differentiation medium contained DMEM supplemented with 10% BCS, 1 µg/mL insulin, 0.5 mM isobutyl methylxanthine (IBMX), and 1.0 µM dexamethasone for 48 hours. Following the induction phase, the medium was replaced with DMEM containing 10% BCS and 1 µg/mL insulin for an additional two days. Subsequently, the cells were cultured in DMEM with 10% Fetal Bovine Serum (FBS) for another six days. At this point, over 90% of the cells exhibited characteristics of mature adipocytes, including the accumulation of fat droplets. On day four of differentiation, the cells were treated with sample extracts in each well. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Measurement of glucose uptake by 2-NBDG

Glucose uptake assays were conducted by seeding 3T3-L1 pre-adipocytes in black and clear flat-bottom 96-well tissue culture plates. The cells were cultured in DMEM supplemented with 10% Bovine Calf Serum (BCS). Adipogenesis was induced by treating the cells with 0.5 mM 3-isobutyl-1-methylxanthine, 2 µg/mL dexamethasone, and 1 µg/mL insulin, with insulin treatment maintained for up to 7 days. On day 10, the cells were starved for 4 hours in glucose-free DMEM and subsequently rinsed three times with D-PBS. They were then incubated in KRPH buffer mixed with insulin, with 100 nM of insulin added per 1 mL of KRPH. The cells were incubated at 37°C for 30 minutes. Following this incubation, 4 µL of

2-NBDG was added to 200 µL of KRPH buffer to achieve a final concentration of 200 mg/mL of 2-NBDG. The cells were incubated at 37°C for an additional hour. The plate was then centrifuged using a microplate centrifuge for 5 minutes at 400 × g at room temperature. The supernatant was aspirated carefully using a pipette.

Next, 200 µL of cell-based assay buffer was added to each well, and the plate was centrifuged a second time for 5 minutes at 400 × g at room temperature. After aspirating the supernatant, 100 µL of cell-based assay buffer was added to each well to initiate the analysis. The fluorescence of the plate was immediately measured using a microplate reader at excitation and emission wavelengths of λ_{ex} = 485 nm and λ_{em} = 535 nm, respectively.

Preparation of cell lysate

The preparation of cell lysate was conducted following the Abcam protocol with some modifications. The cell culture plate was placed on ice, and the cells were washed with ice-cold PBS. After aspirating the PBS, ice-cold RIPA buffer mixed with a protease inhibitor cocktail was added to each well. Specifically, 15 µL of the lysis buffer containing the protease inhibitor was pipetted into each well. The adherent cells were scraped off the dish using a pipette tip and mixed gently by pipetting up and down to ensure thorough suspension. The cell suspension was then carefully transferred into a pre-cooled centrifuge tube and incubated on ice for 1 hour at 4°C. Following incubation, the cells in the centrifuge tube were centrifuged at 12,000 × g for 20 minutes at 4°C. After centrifugation, the tubes were gently removed from the centrifuge and placed back on ice. The supernatant was carefully collected and transferred to a fresh tube, which was kept on ice prior to conducting BCA analysis according to the manufacturer's instruction.

Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics version 22. The results for the percentage of crude yield extract, *in vitro* cytotoxicity of 3T3-L1 cells, and relative 2-NBDG uptake were analysed using one-way analysis of variance (ANOVA). Tukey's Honestly Significant Difference (HSD) test was employed to determine homogeneous subsets within the collected data. Statistical significance was set at a 95% confidence interval, with p-values less than 0.05 considered significant.

RESULTS AND DISCUSSION

Percentage of yield extraction

Figure 2 displays the percentage of extract yield of *O. stamineus* obtained using four different solvents: water (H_2O), ethyl acetate ($C_4H_8O_2$), ethanol (C_2H_6O), and hexane (C_6H_{14}). The yield percentage was calculated based on the crude yield extract obtained after the maceration extraction process. This process included an evaporation step conducted at 40°C to minimize potential degradation of the samples. According to Figure 2, the yield of extract from the solvents is in the following increasing order: hexane < ethyl acetate < ethanol < water. The analysis indicates that the percentage of crude yield in water is the highest among the tested solvents. However, statistical analysis shows that the percentage of crude extract yields from the four solvents is not significantly different ($p < 0.05$). This suggests that the major phytochemicals in *O. stamineus* are primarily high in polarity and soluble in water. Markom *et al.* (2007) reported similar findings in their study of *Phyllanthus niruri*, where the highest yield was also obtained from water due to its greater polarity compared to the other solvents.

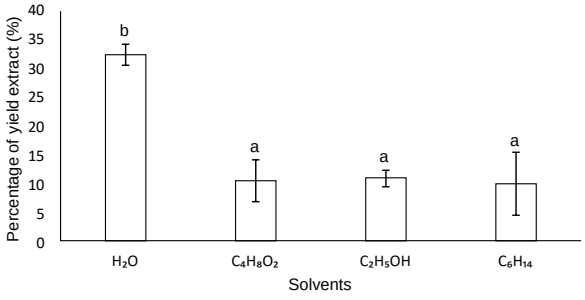

Figure 2. Yield of extraction of *O. stamineus* from different solvents. Error bars represent standard error of triplicates (n=3). Same alphabet on top of the bars indicate that the means of percentage of crude yield extract do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

Although both ethanol and water contain hydroxyl groups that can form hydrogen bonds with solutes, water is more effective in extracting these solutes due to its higher polarity and shorter chain structure compared to other solvents. This difference explains the significant variation observed between the extraction yields of water and ethanol, with water yielding approximately three times more extract than ethanol. In contrast, Markom *et al.* (2007) reported a two-fold difference in yield when using water for extracting *Phyllanthus niruri*, while Xu and He (2007) found a four-fold difference between the extraction yields of water and ethanol for *Pueraria lobata*. These two plants are compared to *O. stamineus* because they share several common compounds and employ the same extraction method. Scientific studies indicate that extraction yields can vary significantly among different medicinal plants, likely due to factors such as the specific phytochemicals present, extraction temperature, and the ratio of solvent to solid.

Extraction plays a crucial role in isolating bioactive compounds from plants. The ultimate goal of extraction is to maximize the yield of high-quality substances that contain a concentrated amount of desirable compounds (Spigno & De Faveri, 2007). The extraction method employed aims to effectively retrieve compounds from plant materials to achieve optimal extraction yield. Murugan and Parimalezhagan (2014) defined extraction yield as the amount of extract recovered in relation to the initial quantity of plant material used. Furthermore, Truong *et al.* (2019) emphasized that extraction yield is measured to evaluate the efficiency of solvents in extracting bioactive compounds from the original material, typically expressed as a percentage (%).

Various extraction methods, such as Soxhlet extraction and maceration, can be employed to isolate compounds from plant materials. The biological activities of plant extracts can differ significantly depending on the extraction method used, highlighting the importance of selecting the appropriate technique (Hayouni *et al.*, 2007). In this study, maceration was chosen as the extraction method because it is suitable for thermolabile constituents and can be performed at room temperature.

Additionally, the choice of extraction solvent is crucial, as it significantly influences the efficiency of extracting bioactive compounds from plant materials and their associated health benefits. In this study, four types of solvents were utilised: water (H_2O), ethanol (C_2H_6O), ethyl acetate ($C_4H_8O_2$), and hexane (C_6H_{14}). These solvents were selected based on their polarity, as outlined in Table 1. The alignment of solvent polarity with the polarity of *Orthosiphon stamineus* phytochemicals directly affects extraction efficiency and bioactive compound composition. Polar solvents, such as water, yield the highest extract due to their ability to solubilize hydrophilic phytochemicals like flavonoids and phenolics. Ethanol, with moderate polarity, shows lower yields, while nonpolar solvents like hexane extract minimal amounts, targeting hydrophobic compounds. This indicates that *O. stamineus* primarily contains polar bioactive compounds, with water being the most effective solvent for their extraction.

Table 1. Properties of organic solvents arranged by decreasing polarity.

Solvent	Formula	Relative polarity[2]
Water	H_2O	1.000
Ethanol	C_2H_6O	0.654
Ethyl acetate	$C_4H_8O_2$	0.228
Hexane	C_6H_{14}	0.009

(Source: Dutkiewicz, 1990)

Cytotoxicity test on 3T3-L1 cell line by MTT assay

Figure 3 illustrates the formazan crystals that were solubilized in DMSO after a four-hour incubation with 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl-tetrazolium bromide (MTT) reagent on the 3T3-L1 cell line treated with varying concentrations of *O. stamineus* extracts. As the concentration of *O. stamineus* extracts increased, the intensity of the purple colour of the formazan crystals diminished. This colour change occurs due to the reduction of tetrazolium salts, indicating a decrease in cell viability.

The dilution of the formazan crystals in DMSO after incubation with the MTT reagent demonstrated that the purple colour became less concentrated with higher concentrations of the sample. This phenomenon can be attributed to a reduction in the number of viable cells present in

the plate as the concentration of the extract increased. Subsequently, the absorbance of the plate was measured using a microplate reader at 570 nm to determine the percentage of viable cells relative to the concentration of *O. stamineus* extracts.

Before conducting the glucose uptake assay to assess the relative uptake of 2-NBDG in 3T3-L1 diabetic cell lines, it was imperative to evaluate the cytotoxicity of the *O. stamineus* extracts to ensure they do not adversely affect cell viability. To achieve this, the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was employed due to its rapid and reliable nature. This assay is based on the capacity of viable cells to reduce tetrazolium salts to formazan crystals, as well as the ability of living cells to incorporate and bind the dye. Additionally, *in vitro* cytotoxicity assessments can identify cellular damage through morphological changes, confirmation of cell integrity, and measurement of cell growth and metabolic properties (Damas *et al.*, 2011; Kasper *et al.*, 2011; Piao *et al.*, 2011; Uboldi *et al.*, 2012). The cytotoxicity of *O. stamineus extracts* was investigated in 3T3-L1 cell lines using two solvents: water and ethanol. Vehicle-treated controls, where cells are exposed to the DMSO without the extract, account for any effects caused by the solvent itself, ensuring that observed changes in viability are due solely to the extracts.

As depicted in Figure 4, the percentage of viable cells is plotted against the concentration of the sample extract. Error bars representing triplicate readings are included to facilitate the assessment of variability. This is crucial for determining the selectivity index of the extract, as it is contingent upon the cytotoxic concentration at 50% (CC50). The MTT assay results indicated that the water extract exhibited toxicity towards Vero cells at a concentration of 0.125 mg/mL (16.7%). In contrast, comparisons of extracts at concentrations ranging from 3.91 x 10^{-3} mg/mL to 6.25 x 10^{-2} mg/mL revealed a significant difference in the water extract ($p < 0.05$), while the ethanolic *O. stamineus* extract showed no significant difference ($p > 0.05$). Overall, these findings indicate that both ethanolic and water extracts are non-toxic to Vero cells.

In contrast, polar extracts from *O. stamineus* did not exhibit any cytotoxic effects on Vero cells, with the exception of the water extract at a concentration of 0.125 mg/mL, which resulted in a cell death rate of 16.7%. The ethanolic extract of *O. stamineus* did not demonstrate any cytotoxic effects on Vero cells. The data indicated that the highest viability for the water extract occurred at a concentration of 7.8 x 10^{-3} mg/mL, while the highest viability for the ethanolic extract was observed at 3.91 x 10^{-3} mg/mL. The variability in absorbance readings may be attributed to chemical interactions between the extracts and the MTT reagent, potentially influenced by the presence of reduction agents in the plant extracts.

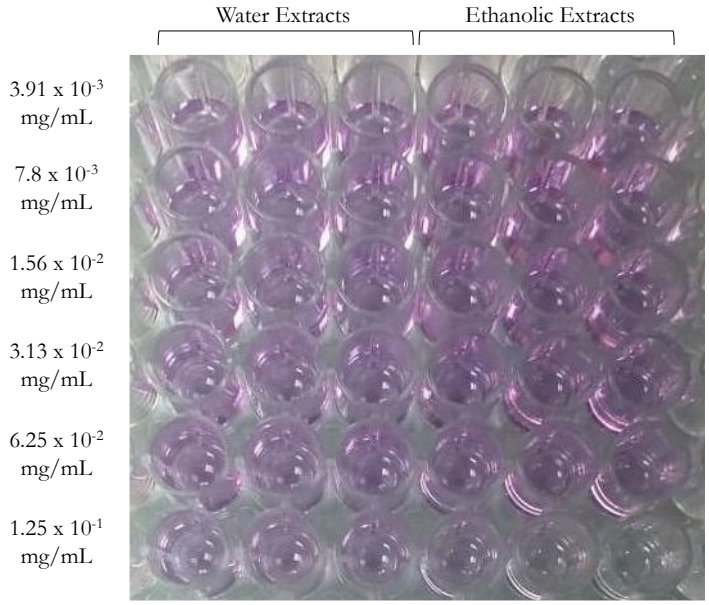

Figure 3. Uptake of crystal violet dye by viable cells observed after 48 hours. The rows indicate the concentrations of the extracts, while the columns represent the triplicates of both water and ethanolic extracts.

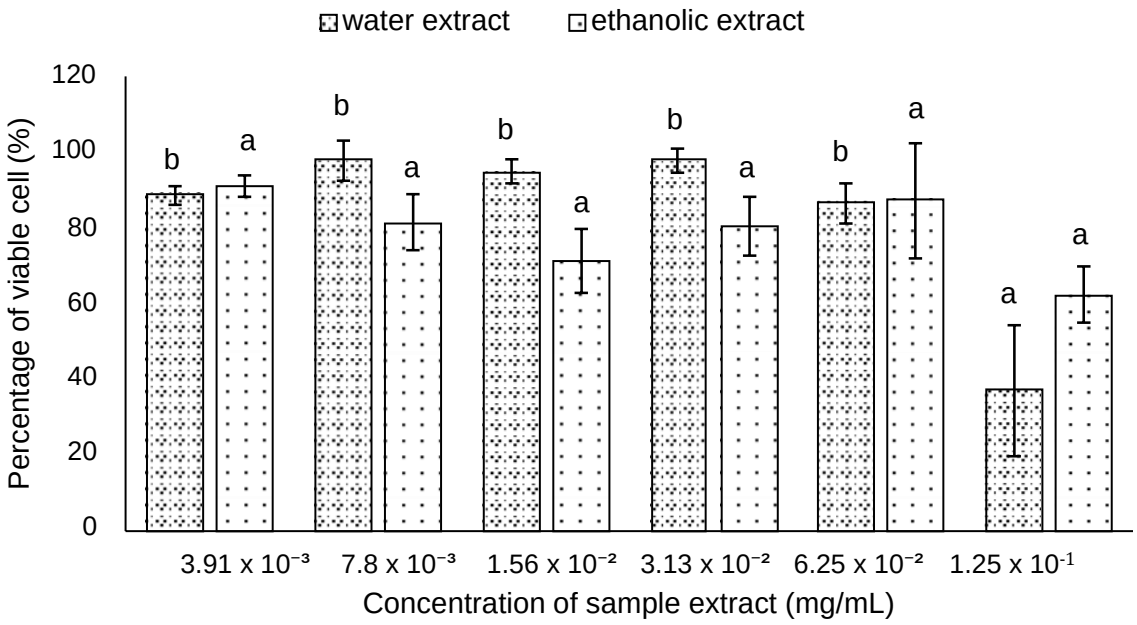

Figure 4. *In vitro* cytotoxicity of *O. stamineus* extracts on the pre-adipocyte 3T3-L1 cell line. Two different extracts were assessed at varying concentrations to evaluate their toxicity on the cells. The error bars represent triplicate readings (n=3). Bars labelled with the same letter indicate that the means of the percentage of viable cells do not differ significantly ($p < 0.05$) based on Tukey's HSD test.

Differentiation of pre-adipocyte 3T3-L1 cell line to diabetic adipocytes for the glucose uptake assay

Figure 5 illustrates the morphology of 3T3-L1 cell lines following 48 hours of treatment with insulin, dexamethasone (DEX), and isobutyl methylxanthine (IBMX). After treatment, confluent 3T3-L1 fibroblasts exhibit adipocyte-like characteristics, indicating successful differentiation. In Figure 5(a), the healthy pre-adipocyte 3T3-L1 cell lines are indicated by the arrow, while Figure 5(b) displays the differentiated adipocytes, characterized by cytoplasm filled with numerous lipid droplets of varying sizes, both at 10X magnification. These results demonstrate that the combination of DEX, IBMX, and insulin effectively induces the differentiation of 3T3-L1 cells into mature adipocytes, which is a prerequisite for conducting the glucose uptake assay.

Prior to differentiation, 3T3-L1 pre-adipocytes resemble typical fibroblasts. Upon induction of differentiation, the cells become thinner and larger, eventually adopting a signet-ring appearance characteristic of mature adipocytes (Yi *et al*., 2000). These morphological changes suggest alterations in the expression of extracellular matrix (ECM) proteins. The ECM serves not only as a structural entity but also plays a critical role in transducing signals that influence various cellular behaviours. Figure 5(b) illustrates the morphology of differentiated adipocytes, which appear as lipid droplets, in contrast to the pre-adipocyte morphology shown in Figure 5(a).

The conversion of pre-adipocytes to fat-rich adipocytes is a complex process that involves comprehensive changes in cellular metabolism, morphology, and gene expression. Although more than 300 proteins have been identified as being significantly altered during the differentiation of 3T3-L1 cells, most of these proteins have yet to be characterized (Sadowski *et al*., 1992). The molecular mechanisms underlying transcriptional regulation during adipocyte differentiation have only been explored for a limited number of genes, with much of the research focusing primarily on transcriptional activation (Yi *et al*., 2000).

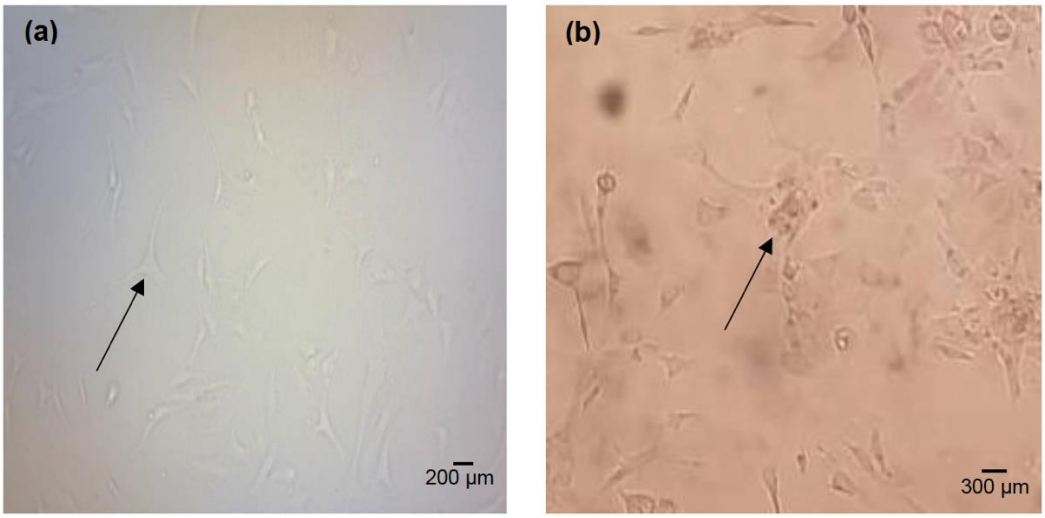

Figure 5. Differentiation of the 3T3-L1 pre-adipocyte cell line into mature adipocytes. (a) Morphology of pre-adipocytes at 70% confluency. (b) Morphology of 3T3-L1 cells after induction to mature adipocytes, showing lipid accumulation as visualized under an Zuess inverted microscope. Pre-adipocytes displayed no obvious lipid droplets, whereas differentiated cells exhibited a round shape characteristic of adipocytes, with nearly all cells containing well-developed lipid droplets (10X magnification).

Furthermore, the 3T3-L1 cell line offers several advantages over freshly isolated cells, such as mature adipocytes. These cells are easier to culture, less costly, and can withstand a higher number of passages while maintaining a homogeneous cell population. Consequently, 3T3-L1 cells provide a consistent response to treatments and variations in experimental conditions.

Relative 2-NBDG uptake on diabetic cell line

The MTT assay results presented in Figure 4 indicate that the extracts demonstrated non-toxicity at concentrations ranging from 3.91 $x10^{-3}$ mg/mL to 6.25 x 10^{-2} mg/mL. Consequently, the glucose uptake assay was performed using two different extracts at concentrations between 0.02 mg/mL and 0.06 mg/mL, as illustrated in Figure 6. These findings suggest that *O. stamineus* extracts have stimulatory effects on 2-NBDG uptake in diabetic cells.

To calculate the relative 2-NBDG uptake, the absorbance from the glucose uptake assay was normalized against the absorbance from the BCA protein assay. This measurement serves to assess insulin sensitivity in the diabetic cell line. Figure 6 shows that the untreated diabetic cells exhibited the highest relative 2-NBDG uptake compared to other concentrations. Notably, the relative 2-NBDG uptake decreased with increasing concentrations of the compounds. Specifically, the glucose uptake corresponding to a concentration of 0.02 mg/mL was the lowest, while the highest glucose uptake was observed at a concentration of 0.06 mg/mL.

The well-differentiated pre-adipocyte 3T3-L1 cell line was utilised to investigate the mechanisms through which plant extracts exert their anti-diabetic effects. The influence of *O. stamineus* extracts on both basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes was assessed using a non-radioactive method for measuring 2-NBDG uptake. Both water and ethanolic extracts of *O. stamineus* demonstrated the ability to enhance insulin-stimulated glucose uptake across a concentration range of 0.02 mg/mL to 0.06 mg/mL. *O. stamineus* is well recognized for its beneficial effects on hyperglycaemia in type 2 diabetes, attributed to its bioactive compounds.

Diabetes mellitus is a metabolic disorder characterized by glucose intolerance and alterations in lipid and protein metabolism. Previous studies have utilized 2-NBDG as a potential agent to evaluate the action of compounds that mimic insulin (Nguyen *et al.*, 2013). Additionally, 2-NBDG is a novel fluorescent derivative of glucose modified with a 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino] group at the C-2 position, with a molecular weight of 342.26 (Yoshioka *et al.*, 1996).

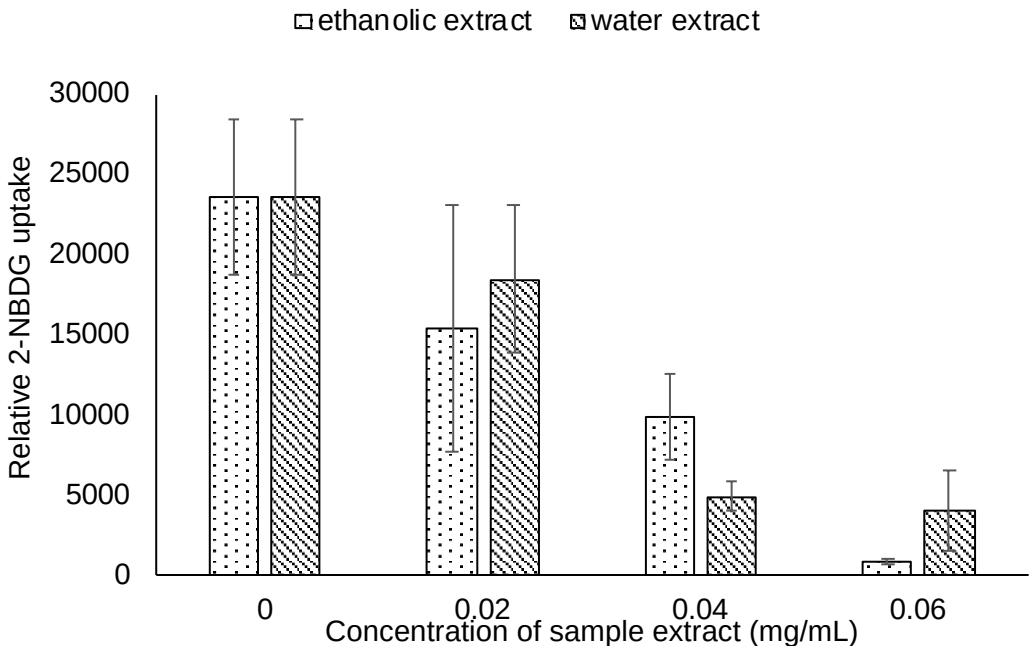

Figure 6. Relative 2-NBDG uptake of O. stamineus extracts on glucose uptake in 3T3-L1 adipocytes following a 10-day treatment period. Adipocyte differentiation was completed on day 10, after which glucose uptake analysis was conducted. Data are expressed as means ± standard error of the mean (SEM) from triplicate readings (n = 3). Identical letters above the bars indicate that the means of relative 2-NBDG uptake do not differ significantly at $p < 0.05$, as determined by Tukey's HSD test.

In this study, the Bicinchoninic Acid (BCA) protein assay was employed to quantify total protein in samples following the glucose uptake assay. The investigation focused on the molecular mechanisms underlying the reduction of lipid accumulation in adipocytes treated with *O. stamineus*, as well as the glucose uptake and transcriptional regulation of the GLUT4 gene in 3T3-L1 cells following treatment with *O. stamineus*. The effects of *O. stamineus* extracts on 2-NBDG uptake were examined in the diabetic 3T3-L1 adipocyte cell line. The glucose uptake observed in 3T3-L1 adipocytes treated with *O. stamineus* extracts may involve mechanisms linked to the regulation of glucose transporter type 4 (GLUT4) translocation. Ten active compounds was identified in *O. stamineus* that exhibits antidiabetic activity partly by neutralising oxidants and promoting GLUT4 translocation to the plasma membrane in skeletal muscle in a rat animal model (Bassalat *et al*., 2023). This suggest that *O. stamineus* extracts demonstrate antioxidant activity, which may be closely linked to their antidiabetic properties and their role in promoting glucose disposal.

One limitation of this study is the absence of a mechanistic analysis to elucidate the specific signaling pathways involved in the observed effects of *O. stamineus* extracts on glucose uptake and antidiabetic activity. Future research should focus on investigating key pathways such as the insulin/PI3K/Akt and AMPK signaling cascades, which are implicated in glucose metabolism and GLUT4 translocation.

CONCLUSION

In this study, the crude extract of *O. stamineus* was obtained using the maceration method with four different solvents: water, ethyl acetate, ethanol, and hexane. The yield percentages of the extracts were determined, revealing an increasing order of extraction efficiency as follows: hexane < ethyl acetate < ethanol < water. Water yielded the highest percentage of extract, followed by ethanol, ethyl acetate, and hexane, indicating that the major phytochemicals in *O. stamineus* leaves are predominantly polar and soluble in water.

Consequently, water was selected as a solvent for further experiments due to its superior extraction capacity compared to ethyl acetate and hexane.

The *in vitro toxicity* of *O. stamineus* extracts was assessed using the MTT assay with water and ethanol extracts. Results indicated that the ethanol extract was non-toxic, exhibiting the highest percentage of viable cells at a concentration of 3.91 x 10⁻³ mg/mL. In contrast, the water extract showed a significant cytotoxic effect at a concentration of 1.25 x 10⁻¹ mg/mL, maybe to the osmotic imbalance, where isotonic characteristics of the extract could disrupt cell membrane integrity and lead to cell lysis. Notably, both extracts maintained cell viability above 50%, as indicated by the cytotoxic concentration at fifty percent (CC_{50}). For future studies, it would be beneficial to compare the Neutral Red Uptake (NRU) assay with the MTT assay to determine the most effective method for evaluating cytotoxic effects. Additionally, using low-passage cell lines is recommended to minimize issues related to cell fragility and detachment during experiments.

Regarding glucose uptake, the relative 2-NBDG uptake was highest at a concentration of 0.02 mg/mL, while the lowest glucose uptake was observed at 0.06 mg/mL. Significant differences were noted between the water and ethanol extracts ($p < 0.05$). Evaluating glucose uptake is a critical aspect of diabetes research, and the use of 2-NBDG as a fluorescent glucose analog offers a rapid and non-radioactive method for measuring glucose uptake, providing a valuable tool for both anti-diabetic drug development and fundamental diabetes research.

Future investigations should focus on elucidating the molecular mechanisms by which *O. stamineus* and its active compounds regulate glucose uptake in adipose and muscle tissues. This study is the first to explore the potential of *O. stamineus* extracts in managing diabetes and related complications using the non-radioactive fluorescent indicator 2-NBDG. The findings underscore the importance of developing glucose-lowering therapies with minimal adipogenic effects, and further research is needed to identify the specific chemical structures responsible for the observed biological activities.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

REFERENCES

Bassalat, N., Kadan, S., Melamed, S., Yaron, T. Tietel, Z., Karam, D., Kmail, A., Masalha, M., Zaid, H. (2023) In Vivo and In Vitro Antidiabetic Efficacy of Aqueous and Methanolic Extracts of Orthosiphon Stamineus Benth. *Pharmaceutics*, 15:945.

Damas, B. A., Wheater, M. A., Bringas, J. S. & Hoen, M. M. (2011). Cytotoxicity comparison of mineral trioxide aggregates and endo-sequence bio-ceramic root repair materials. *Journal of Endodontics,* 37:372-375.

Fenton, J. J. (2002). Toxicology: A case oriented approach. New York: CRC Press.

Hayouni, A. E., Abedrabba, M., Bouix, M., & Hamdi, M. (2007). The effects of solvents and extraction method on the phenolic contents and biological activities *in vitro* of *Tunisiam quercus coccifera* L. and J*uniperus phoenica* L. fruit extracts. *Food Chemistry,* 105:1126-1134.

Kasper, J., Hermanns, M. I., Bantz, C., Maskos, M., Stauber, R., Pohl, C., Unger, R. E., & Kirkpatrick, J. C. (2011). Inflammatory and cytotoxic responses of an alveolar-capillary co-culture model to silica nanoparticles: Comparison with conventional monocultures. *Particle and Fibre Toxicology,* 8:1-6

Lee, H.J., Choi, Y.J., Park, S.Y., Kim, J.Y., Won, K.C., Son, J.K., Kim, Y.W. (2015). Hexane Extract of Orthosiphon stamineus Induces Insulin Expression and Prevents Glucotoxicity in INS-1 Cells. *Diabetes & Metabolism Journal*, ;39(1):51-8.

Markom, M., Hassan, M., Wan Daud, W. R., Singh, H., & Jahim, J. (2007). Extraction of hydrolysable tannins from *Phyllanthus niruri* linn: Effects of solvents and extraction methods. *Separation and Purification Technology*, 52:487-496.

Mohamed, E. A. H., Siddiqui, M. J. A., Ang, L. F., Sadikun, A., Tan, S. C., Chan, S. H., Asmawi, M. Z., & Yam, M. F. (2012). Potent α-glucosidase and α-amylase inhibitory activities of standardized 50% ethanolic extracts and sinensetin from *Orthosiphon stamineus* benth as anti-diabetic mechanism. *BMC Complementary and Alternative Medicine*, 12:*12*, 1-7.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65:55-63.

Muhammad, Z., & Mustafa, M. A. (1994). Traditional Malay Medicinal Plants. Kuala Lumpur: Penerbit Fajar Bakti Sdn. Bhd.

Murugan, R., & Parimelazhagan, T. (2014). Comparative evaluation of different extraction methods for antioxidant and anti-inflammatory properties from *Osbeckia parvifolia* arn. An in vitro approach. *Journal of King Saud University – Science*, 26:267-275.

Nguyen, D., Samson, S. L., Reddy, V. T., Gonzalez, E. V., & Shekhar, R. V. (2013). Impaired mitochondrial fatty acid oxidation and insulin resistance in aging: Novel protective role of glutathione. *Aging cell*, 12(3):415-425.

Piao, M. J., Kang, K. A., Lee, I. K., Kim, H. S., Kim, S., Choi, J. Y., Choi, J. & Hyun, J. W. (2011). Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. *Toxicology Letters,* 201:92-100.

Rao, N. K., Bethala, K., Sisinthy, S. P., & Rajeswari, K. S. (2014). Antidiabetic activity of *Orthosiphon stamineus* benth roots in streptozotocin induced type 2 diabetic rats. *Asian Journal of Pharmaceutical and Clinical Research*, 7(1):149-153.

Sadowski, H. B., Wheeler, T. T., & Young, D. A. (1992). Gene expression during 3T3- L1 adipocyte differentiation: Characterization of initial responses to the inducing agents and changes during commitment to differentiation. *The Journal of Biological Chemistry*, 266(7):4722-4731.

Spigno, G., Tramelli, L. & De Faveri, D.M. (2007) Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *Journal of Food Engineering*. 81:200-208.

Sriplang, K., Adisakwattana, S., Rungsipipat, A., & Yibchok-Anun, S. (2007). Effects of *Orthosiphon stamineus* aqueous extract on plasma glucose concentration and lipid profile in normal and streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology*, 109:510-514.

Truong, D. H., Nguyen, D. H., Anh Ta, N. T., Bui, A. V., Do, T. H., & Nguyen, H. C. (2019). Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and *in vitro* anti-inflammatory activities of *Severinia buxifolia*. *Journal of Food Quality*, 2019:1-9.

Uboldi, C., Giudetti, G., Broggi, F., Gilliland, D., Ponti, J. & Rossi, F. (2012). Amorphous silica nanoparticles do not induce cytotoxicity, cell transformation or genotoxicity in Balb/3T3 mouse fibroblasts. *Mutation Research,* 745:11-20.

Vijayalakshmi, K., & Selvaraj, C. I. (2018). Cell line cytotoxicity, antiadipogenic and glucose uptake activity of *Sarcostemma brevistigma* Wight. & Arn. *Molecular Biology Reports,* 45:2555-2561.

Wang, X., Zhao, W., Zhang, X., Wang, Z., Han, C., Xu, J., Yang, G., Peng., J., Li, Z. (2023). An integrative analysis to predict the active compounds and explore polypharmacological mechanisms of *Orthosiphon stamineus* Benth. *Computers in Biology and Medicine*, 163: 107160.

Xu, H. N., & He, C. H. (2007). Extraction of isoflavones from stem of *Pueraria lobata* (willd.) Ohwi using n-butanol/water two-phase solvent system and separation of daidzein. *Separation and Purification Technology*, 56:85-89.

Yam, M. F., Lim, V., Salman, I. M., Ameer, O. Z., Ang, L. F., Rosidah, N., Abdulkarim, M. F., Abdullah, G. Z., Basir, R., Sadikun, A., & Asmawi, M. Z. (2010). HPLC and anti-inflammatory studies of the flavonoid rich chloroform extract fraction of *Orthosiphon stamineus* leaves. *Molecules*, 15:4452-4466.

Yi, T. G., Lee, B. H., Park, R. W., & Kim, I. S. (2000). Transactivation of fibronectin promoter by HTLV-1 tax through NF-κB pathway. *Biochemical and Biophysical Research Communications*, 276:579-86.

Yoshioka, K., Takahashi, H., Homma, T., Saito, M., Oh, K. B., Nemoto, Y., & Matsuoka, H. (1996). A novel fluorescent derivative of glucose applicable to the assessment of glucose uptake activity of *Escherichia coli*. *Biochimica et Biophysica Acta*, 1289(1):5-9.

Zebisch, K., Voigt, V., Wabitsch, M., & Brandsch, M. (2012). Protocol for effective differentiation of 3T3-L1 cells to adipocytes. *Analytical Biochemistry*, 425(1):88-90.