Exploring the chemistry and evaluation of the antidiabetic potential of chemicals from Spermacoce hispida L., (Nathaichoori) on 3T3-L1 cells- an in vitro approach.

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Abstract

We assessed the phytoconstituents of aqueous and ethanolic extracts of Spermacocehispida L leaves. Ethanolic extract of Spermacocehispida L. subjected to GC-MS analysis elucidated the occurrence of 55 bio-active chemical compounds. Effective inhibitory activity of □- amylase, and □-glucosidase was shown by both the extracts. Glucose absorption capacity on the 3T3 L1 cell line and glucose diffusion assay showed the capacity of the chemicals from plant extracts to bind to glucose and retard its diffusion through the dialysis membrane. MTT assay results suggested no cytotoxic effect by both the plant extracts on the adipocyte cells. Results suggested the antidiabetic potential of Spermacocehispida L chemical extracts and in-vivo studies are needed for future clinical implications.

Keywords: Diabetes mellitus, alpha-amylase, alpha-glucosidase, inhibition, Spermacocehispida L.

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1. INTRODUCTION

Diabetes mellitus is a grave metabolism-related disorder that significantly affects well-being, living standards, and life duration [1]. It has multiple etiologies with significant consequences, both acute and chronic. It is categorized into two major categories viz. type 1 diabetes relies on insulin levels while type 2 is independent of insulin. The disease and its after-effects have serious consequences on the lives of people in both developed and developing nations causing social and economic struggles. Diabetes mellitus and its complications affect people both in developing and developed countries, leading to the socioeconomic challenge. Nearly 25% of people in the world suffer from this disease [2]. It is marked by high blood glucose levels along with altered metabolism of macromolecules due to abnormal release of insulin in terms of quantity and action. The persistently high levels of blood glucose cause organ impairment over time, and the worst affected are the kidneys, nerves, heart, eyes, and blood vessels [3].

Various classes of oral hypoglycemic drugs are available, namely Biguanides (e.g., metformin), sulfonylureas (e.g., glimepiride/glibenclamide), thiazolidinediones (e.g., pioglitazone), dipeptidyl peptidase IV inhibitors (e.g., Vildagliptin), meglitinides (e.g., repaglinide), SGL2 inhibitors and α-glucosidase inhibitors. These exert their antidiabetic effect by different mechanisms. All these drugs, along with insulin, constitute the prime treatment regimen, but none of these could nullify the damage associated with diabetes and also have serious side effects. Therefore, necessitates discovering alternative sources of novel antidiabetic agents [4]. In the last three decades, a lot of progress has been made in the treatment of diabetes using oral antidiabetic agents, but still, the results are far from perfect. Demerits associated with oral hypoglycemic agents include the development of drug resistance, adverse effects, reduction in efficacy as well as toxicity. It has been shown that sulfonylureas lose their efficacy in nearly 44% of patients after 6 years of treatment [5, 6]. Various side effects and drawbacks of using synthetic antidiabetic drugs resulted in the quest for novel agents for treatment from natural sources [7].

Plants-based products have gained a lot of focus in upcoming drug discovery programs due to the associated benefits of easy procurement, less expenditure, and minimal after-effects [8, 9]. These medicinal plants have a great deal of pharmacologically significant bioactive chemicals and do not possess harmful after-effects. A large number of plants have been utilized for having blood sugar lowering the potential for years together. To overcome the prevailing medicines cost particularly in many developing countries, medicinal plants have been widely used as a drug used to treat diabetes mellitus. Not only in developing countries but also nowadays they are recommended throughout the world for the cure of various health-related issues, including diabetes [10]. In the current study, we have analyzed the antidiabetic activity of aqueous and ethanolic extracts of leaves of a medicinal plant, *Spermacocehispida*, as well as characterized the phytoconstituents present in the ethanolic extract by GC-MS analysis.

*Spermacocehispida*linn, usually called the "shaggy button weed," belongs to the family Rubiaceae (Figure 1) and is a widely utilized medicinal plant worldwide [11]. In the Siddha medicine system, this plant has been used for treating various conditions like diabetes mellitus, diarrhea, etc. The whole plant is used for various medicinal properties [12]. Seeds of this plant are crushed and given orally for the treatment of stomach problems, including diarrhea and dysentery [13]. Plants extracted from this plant have been found to contain phytochemical constituents such as saponins, tannins, phenolics, steroids, essential oils, flavonoids, and terpenoids [13]. Crude extracts along with these purified chemical entities of the plant, have been used for various medicinal properties. Other extensively studied phytochemicals and active ingredients of Spermacocehispida include Borreline, β-sitosterol, Ursolic acid, and Isorhmnatin with various activities. Spermacocehispida has been explored for its various medicinal activities, including antioxidant, anti-inflammatory, analgesic, anti-fungal, hypolipidemic, hepatoprotective, and anti-cancer potential [13-

16]. So far, its antidiabetic actions are not much described. Therefore, keeping this in view, in this study, we evaluated the aqueous and ethanol

extract of leaves of Spermacocehispida as a drug to treat diabetes mellitus and characterized the phytochemical components existing in the aqueous extract. We also evaluated if these extracts show any cytotoxicity to further assess the safety of using these extracts for future clinical purposes.

2. MATERIALS AND METHODS

The plants were collected from Thirumandangudi, Thanjavur district, Tamil Nadu, India. The identity of the plant was authenticated at the XXXX, Voucher No. SRC-SASTRA-0022. The plant material (whole plant) was shade-dried for 15 days and powdered. 3T3-L1 cells were procured from XXXX, and cultured in Dulbecco's Modified Eagle's Medium (DMEM). The selected plant species is shown in Figure 1a.

2.1. In-vitro glucose uptake potential of ethanol extract of whole plant of *Spermacocehispida*

Glucose uptake activity in 3T3-L1 cells of ethanol extract of the whole plant of Spermacocehispida L. was evaluated. Cell cultures grown in DMEM with 2% FBS for 4-6 days and having 70-80% confluency in 40 mm Petri plates were made to differentiate. It was confirmed by perceiving the presence of multi-nucleation within cells. The later were deprived of serum overnight. Subsequently, the cells were washed with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer) once and incubated with KRP buffer with 0.1% BSA for 30min at 37°C and were treated with different concentrations (25, 50, 100, 200, and 400 µg) of ethanol extract of Spermacocehispida L. for 30 min along with negative controls at 37°C. D-glucose solution was added simultaneously to each well and incubated at 37°C for 30 min. After incubation, the uptake of the glucose by the 3T3 L1 cells was terminated by aspiration of solutions from wells and washing thrice with ice-cold KRP buffer solution. Cells were lysed with a 0.1 M solution of NaOH, and an aliquot of cell lysates was used to measure the cell-associated glucose. The glucose levels in cell lysates were measured using a glucose assay kit.

2.2. Glucose diffusion inhibition assay

The experimental setup was prepared by loading 1 ml of extract (500 µg/ml) and 1 ml of glucose solution (22 mM in 0.15 M NaCl) into the dialysis bag. The control bag was loaded with 1 ml of 0.15 M NaCl, 1 ml glucose, and 1 ml distilled water. The dialysis bag was tied at both ends using a thread and placed in a 200 ml beaker containing 45 ml of 0.15 M NaCl. The beakers were placed in an orbital shaker at room temperature. The glucose concentration of the external solution was measured by using a glucose reagent every half an hour up to 3 h. Triplicates of the experiment were done to get concordant values [17].

The GDRI (Glucose diffusion retardation index) was calculated with the following formula:

$$\textbf{GDRI (\%)} = \frac{100 - \text{Glucosecontent with the addition of sample}(\text{mg dl}^{-1})}{\text{Glucosecontent of the control}(\text{mg dL}^{-1})} \times 100$$

Glucose adsorption assay [18]

1 gram of plant extract was prepared and poured into a test tube containing 100 ml of different concentrations (5, 10, and 20 mM) of glucose solution. The amount of glucose present in the solution was measured initially. Each of these mixtures containing the glucose solution along with plant extract was mixed well, stirred, and then placed in a shaker at 37°C for 6 h. After incubation, the mixture was centrifuged for 20 min at 4800 rpm. After that, a glucose assay kit was used to quantify the glucose levels of the supernatant. The amount of glucose adsorbed to the plant extract was determined by the given formula:

G1-G6

Glucose bound= ----------□ volume of sample

Weight of sample

G1 - Glucose concentration of original solution

G6 - Glucose concentration after 6 h

3. Results

The preliminary phytochemical tests of the various extracts of Spermacocehispida L. revealed the presence of many phytochemical constituents. The dry powder contained glycosides, terpenoids, tannins, flavonoids, carbohydrates, amino acids, and phenolic compounds. The ethanolic extract of the plant contained alkaloids, steroids, carbohydrates, and quinones. The aqueous extract of the plant contained alkaloids, terpenoids, carbohydrates, and quinones (Table 1).

S.No.	Phytoconstituents	Powder	Aqueous	Ethanol
1	Alkaloids	-	+	+
2	Glycosides	+	-	-
3	Terpenoids	+	+	-
4	Steroids	-	-	+
5	Saponins	-	-	-
6	Flavonoids	+	-	-
7	Tannins	+	-	-
8	Carbohydrates	+	+	+
9	Amino acids	+	-	-
10	Phenolic compounds	+	-	-
11	Phlobatannins	-	-	-
12	Quinones	-	+	+
13	Anthocyanins	-	-	-

Table 1. Qualitative Phytochemical Analysis of *Spermacocehispida*leaves extract

Our result showed that both the extracts of *Spermacocehispida* L. effectively inhibited the glucose diffusion across the dialysis membrane when with the control. From 1 h to 3 h, the rate of glucose diffusion inhibition was shown to rise. The aqueous extract had a higher inhibitory potential when compared to the ethanolic extract. From the results obtained, it was clear that the extracts of *Spermacocehispida* L. possessed hypoglycemic potential and acted by inhibiting the entry of glucose, thereby controlling the post-prandial blood glucose. Therefore, it could be a potential drug for the management of diabetic mellitus.

The observations further deciphered that the aqueous and ethanolic extracts of *Spermacocehispida L.* had a significant glucose adsorption capacity (Table 2). The aqueous extract deciphered the maximum glucose adsorption capacity of 56% at 50 mM of glucose concentration when compared to the ethanol extract, which showed only 34.54% glucose

adsorption capacity at 50 mM glucose concentration. The glucose adsorption capability of the plant extract was found toincrease with the increased glucose content (Figure 1b).

Table1. Sample application

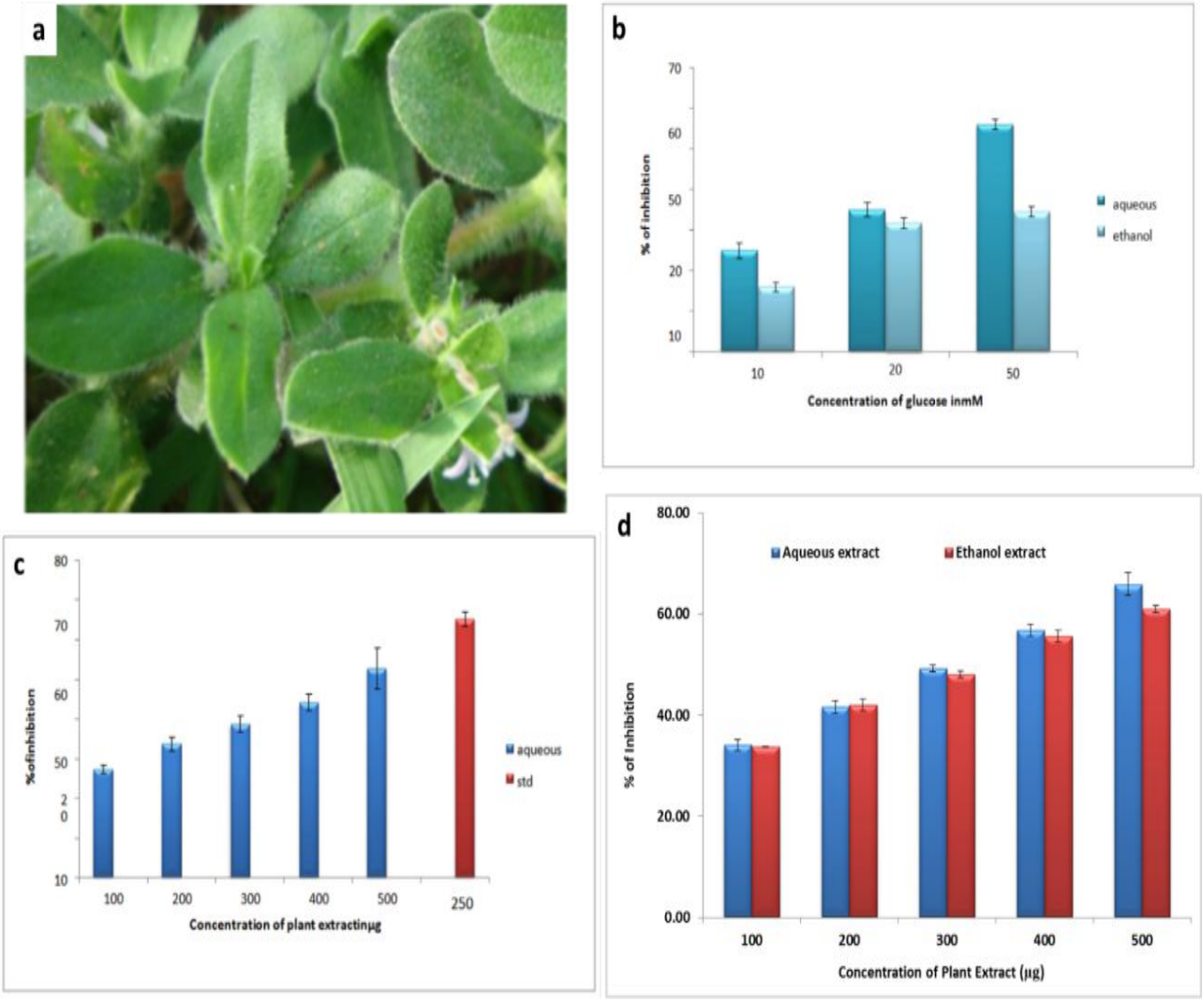

Figure 1. (a) *Spermacocehispida* (local name: nathaichoori). (b) Effect of *Spermacocehispida* L. extracts on glucose uptake capacity on 3T3-L1 cells. (c) Inhibitory effect of *S.hispida* L on α glucosidase activity. (d) Effect of *S. hispida*L. extracts on alpha amylase

The values are expressed as mean ± SD, n=3.

Table 2. Effect of *Spermacocehispida*L. extracts on glucose adsorption.

Concentration of glucose	Concentration of plant extract	% of glucose adsorption	
		Aqueous extract	Ethanol extract
10 mM	1%	24.99±1.92	16.02±1.10

20 mM		35.13±1.80	32.83±1.37
50 mM		56.09±1.22	34.54±1.27

The study further observed that both aqueous and ethanol extract of the *S.hispida L*. effectively inhibited the alpha-amylase enzyme activity. In particular, the aqueous extract showed a slightly high inhibitory activity of 65.90% than the ethanol extract, which showed 60.98% inhibition of the enzyme at a concentration of 500 µg/ml. The IC50 value of aqueous extract was calculated to be 335.57 µg/ml, and that of ethanolic extract was calculated to be 349.65 µg/ml. The results were compared with the standard drug acarbose, which showed a slightly higher percentage of inhibition at a concentration of 250 µg/ml, i.e., 68.14±2.27% (Figure 1d). Our results indicated that the aqueous extract of *S.hispida* L. showed significant inhibitory activity on alpha-glucosidase. The extract has the highest inhibition activity of 52.68% at 500 µg/ml. The IC50 value of aqueous extract was 423.72 µg/ml (Figure 1c).

The potential of the plant extract to stimulate the absorption of glucose present in the external solution was assessed using 3T3 –L1 adipocyte cells. The plant extracts enhanced the glucose absorption in 3T3-L1 adipocytes, according to our findings. The glucose uptake was high as 57% at a concentration of 400mg of ethanolic extract, as compared to the control (Figure 3a). The above results showed that both the extracts of *S.hispida* L. did not induce any cytotoxic effect at varying concentrations and the cytotoxic effect was less than 1% (Figure 3b).

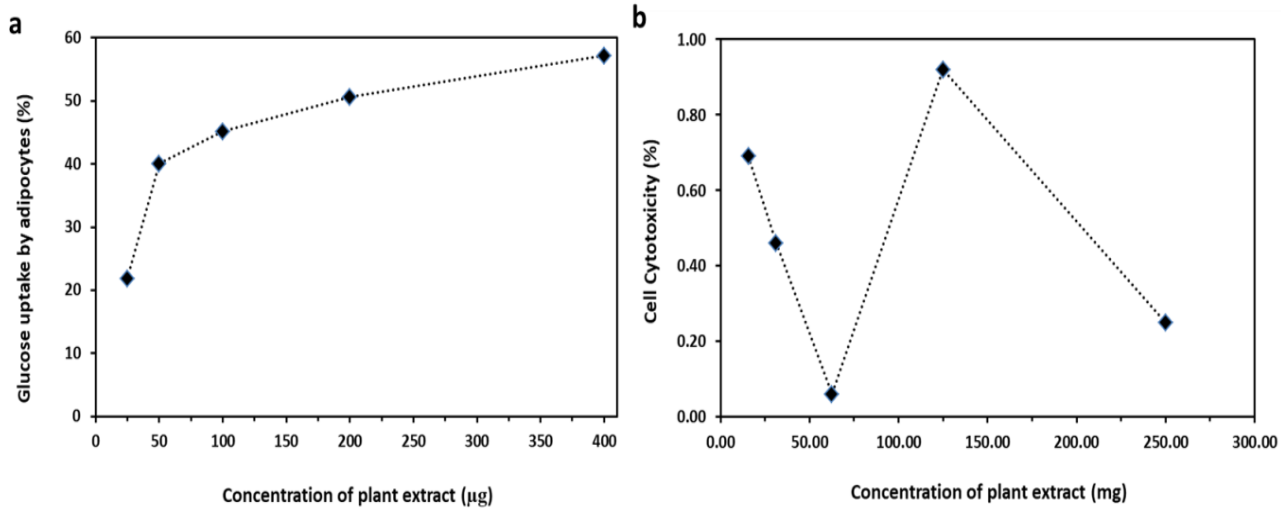

Figure 3. (a) Effect of ethanol extract of *S.hispida* on glucose uptake by adipocytes. (b) Effect of S.hispida L on viability 3T3-L1 cell lines

The findings of GC-MS analysis of ethanolic fraction of *Spermacocehispida L*. highlighted many chemicals, which have been listed in Table 3. The compounds were identified using mass spectrometry attached with GC.

Sl no	Compound name and Mol. wt	Formula	Retention time	Area	Area %

Table 3. GC MS analysis of ethanol extract of *S.hispida*leaves

1	**Name:** Benzene,1,3-dimethyl **Mol. wt:**106	C_8H_{10}	4.3	5919408	3.01
2	**Name:**2H-1-Benzopyran, 6,7-dimethoxy-2,2-dimethyl- **Mol. wt**:220	$C_{13}H_{16}O_3$	21.27	6108062	3.11
3	**Name:** n-Hexadecanoic acid **Mol. wt:** 256	$C_{16}H_{32}O_2$	26.696	3E+07	15.01
4	**Name:** Phytol **Mol. wt:** 296	$C_{20}H_{40}O$	28.945	2.9E+07	14.53
5	**Name:**9,12Octadecadienoicacid(Z,Z)- **Mol. wt:** 280	$C_{18}H_{32}O_2$	29.339	4916599	2.5
6	**Name:**9-OCTADECENAL,(Z)-**Mol. wt:**266	$C_{18}H_{34}O$	29.445	1.1E+07	5.6
7	**Name:**9-Octadecenoic acid,(E)-**Mol. wt**:282	$C_{18}H_{34}O_2$	29.515	5880705	2.99
8	**Name:**Linoleic acid ethylester **Mol. wt:** 308	$C_{20}H_{36}O_2$	29.687	5221350	2.66
9	**Name:** EthylOleate **Mol. wt:** 310	$C_{20}H_{38}O_2$	29.785	4693691	2.39
10	**Name:**Oxalicacid,mono-(5-[(2-bromophenyl)(2,2-dimethylpropionyloxy)methyl]-7,8-dihydro-5H-[1,3]dioxolo[4,5-g]isoquinolin-6-yl)ester **Mol. wt:** 535	$C_{24}H_{26}BrNO_8$	34.601	4510196	2.29
11	**Name:** Tetradecanal **Mol. wt:** 212	$C_{14}H_{28}O$	34.852	3777886	1.92

4. Discussion

The presence of phytochemicals such as flavonoids, terpenoids, saponins, and alkaloids present in these medicinal plants is believed to contribute to their antidiabetic potential [19]. Innumerable plants and their extracts have been tried with moderate success but with inherent difficulties in commercial use [5].

Spermacocehispida of the Rubiaceaefamily is known to have therapeutic potential against obesity, diarrhea, and dyslipidemia) [20, 21]. However, its antidiabetic properties have not been investigated. Herein, we explored the antidiabetic potential of extracts of leaves of this plant. As published previously, the extraction of numerous phytoconstituents was also conducted [22]. To date, only a couple of researchers have enumerated the potential of plant extracts in the context of the movement of glucose within the intestinal tract via diffusion. Herein, we studied the effect of both extracts on the glucose movement across the dialysis membrane in the external solution. This is a very easy way to assess the contributing parameters that regulate in vitro glucose absorption. Gallagher et al. used this

method to evaluate the glucose diffusion suppression capacity of different plants and found that agrimony and avocado have a stronger suppression effect on glucose transport across the membrane (more than 60 percent) [17]. The present study also demonstrated the inhibitory potential of both aqueous and ethanolic extract of leaves of *Spermacocehispida*on glucose transport across the membrane. This result indicated that the antidiabetic properties of these extracts might be via reducing glucose absorption in vivo. In the glucose adsorption test, the ability of the plant extract to bind with the glucose molecule was evaluated. Our data suggested that both the aqueous and ethanolic extracts have strong glucose adsorption capacity. Das et al. (2015) have found this property in the extracts of *Terminalia bellirica* [23].

Carbohydrate hydrolyzing enzymes, viz. α-amylase, and α-glucosidase, are known to digest the dietary starch and degrade the oligosaccharides to glucose which results in the surge in post-prandial glucose. Therefore, studying the inhibition of the activity of both these enzymes is one of the primary approaches to evaluating the management of the hyperglycemic condition in diabetes patients. Acarbose is the most common drug prescribed for diabetes and is a glucosidase inhibitory drug [24]. Various studies have suggested that medicinal plants and their phytochemicals show antidiabetic therapeutic potential via Inhibitory effects against a-amylase and a-glucosidase [25, 26]. Therefore, it could be postulated that inhibition of these enzymes by the plant extract demonstrates its antihyperglycemic effects. Our study found the potential inhibitory effect of both aqueous and ethanolic extracts on the activity of α-amylase and α-glucosidase, which further ascertained their antidiabetic potential.

We also assessed the cytotoxic effect of both extracts, if any, using 3T3-L1 adipocyte cells. Data suggested that these extracts did not have any cytotoxic effect on the cells, establishing that both aqueous and ethanolic extracts are specific in their antidiabetic property and do not cause any cytotoxic effect to healthy cells indicating their safety for clinical use in future.

Our overall data suggested that both the extracts of plant Nathaichoori show significant antidiabetic potential with minimal toxicity to the cells suggesting that both the extracts are safe to use.

The GC MS analysis of the ethanolic extract identified around 55 compounds which included beneficial omega-3 fatty acids [27]. These omega-3 fatty acids have been reported to help prevent glucose intolerance and for treating diabetes [28, 29]. It could be inferred that these omega-3 fatty acids in the ethanolic extract might have contributed to its antidiabetic potential.

Although the findings of our study explored the antidiabetic potential of the aqueous and ethanolic extract of leaves of *Spermacocehispida*, this study had various limitations. The major limitation of the study is that there was neither an animal nor a human experiment conducted to further establish our findings. Animal studies are much needed, followed by human experiments to transfer the use of these extracts from bench side to bedside, i.e., for clinical use.

Even though numerous studies of plants and antidiabetic efficiencies exist, ours is unique with enzyme inhibitions and glucose diffusion assays. We are the first to report the plant on cell culture and establish its safety.

5. Conclusion

To conclude, the aqueous and ethanolic chemical extracts of the plant *Spermacocehispida*known as Nathaichoori, showed a potential antidiabetic effect. The inhibitions of the enzymes alpha-amylase and alpha-glucosidase are noteworthy for gaining market entry as antidiabetic agents. The results of the glucose diffusion and adsorption assays were favorable as antihyperglycemic drugs. In 3T3-L1 cell studies, we have found no toxic effect of the extracts. The GC MS analyses showed the presence of numerous chemicals like omega-3 fatty acids. Overall, further extensive studies of these chemical extracts could lead to a safe potential antidiabetic agent at a low cost. An

individual chemical analyses on the effect is the need of the future.

CONFLICT OF INTEREST

The authors have no competing interests to declare that are relevant to the content of this article.

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NA

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