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PHARMACOLOGICAL EVALUATION OF HERBAL EXTRACTS FOR THEIR *INVITRO* HYPOGLYCEMIC ACTIVITY

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ABSTRACT

Diabetes mellitus is a metabolic disorder and the management is an important criterion for pharmacotherapy. The medicinal plants play very important role in preventing the progress of the disease. Present study deals with screening of polyherbal extracts using Invitro techniques for its antidiabetic activity. The plants used in the study are *Eugenia jambolana* and *Cinnamomum zeylenicum*. The plants were extracted using direct soxhlet method with various solvents. The polyherbal extracts were subjected to *invitro* evaluation using cell culture assays, alpha-glucosidase inhibition and antioxidant property. The cell lines 3T3L1 C2C12 and HepG2 were used to measure the uptake of glucose. The results showed that the glucose uptake was more in APKJ-004 polyherbal extract when compared with other extracts. α-glucosidase inhibitory activity was found in all the extracts and they were comparable to the standard acarbose and the highest inhibitory activity was found in the polyherbal extract APKJ004 (IC_{50}147.2μg/ml). The *invitro* antioxidant potential revealed that the extract APKJ004 is more potent and is comparable to the standard. Based on the *invitro* evaluations the unique combination of glucose uptake activity and α glucosidase inhibition potential of extract APKJ-004 with prominent hypoglycemic activity will aid in the treatment of diabetis mellitus. The i*nvivo* experiments to prove the results obtained in *invitro* studies are in progress.

Key words: Antidiabetic activity, 3t3-L1, C2C12, HepG2, polyherbal extract, Glucose uptake, α-glucosidase.

INTRODUCTION

Diabetes mellitus is a chronic disease characterized by elevated blood glucose levels, disturbances in the carbohydrate, fat and protein metabolism (Apparao *et al.,* 2003). Over several years diabetes mellitus has become a major health problem worldwide; reaching epidemic proportions (Modak *et al.,* 2007). Diabetes mellitus is considered to be a serious issue in many countries and traditional methods using medicinal plants to control diabetes is gaining momentum (Pari and Ramakrishna, 2001). The synthetic hypoglycemic agent/s does produce serious side effects (Kameshwara Rao *et al.,* 2001) and whereas drug derived from medicinal plants are frequently consider being safe & cost effective. Herbal preparations are used to treat diabetes, as an alternative therapy but their reported hypoglycemic effects are multifarious. Hence there is a need to search for safer hypoglycemic agent/s from plant origin which will address the issue. The Literature survey revealed that several thousands of plants showed the antidiabetic activity but the study lacks the proper scientific validation and systematic evaluation (Jia *et al*., 2009).

The present study was conducted on plant species *Eugenia jambolana and Cinnamomum zeylenicum* to evaluate its antidiabetic activity by using *invitro* techniques. The polyherbal preparations were made with aqueous and organic solvents in single and as well in combinations with different proportions and were used to screen the antidiabetic potential by using Invitro evaluations.

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MATERIALS AND METHODS

The plant materials *Eugenia jambolana and Cinnamomum zeylenicum* were collected from Acharya N.G Ranga University and was authenticated by Dr.P.Jayaraman taxonomist at Plant Research Centre, Chennai and the specimen was preserved. Collected material was shade dried, chopped and grinded to powdered form. The powdered stem bark of *Cinnamomum zeylenicum* and seeds of *Eugenia jambolana* was extracted with ethyl alcohol (95%) and water at temperature (65-75^{0}C) using direct soxhlet method (Palanimuthu *et al.,* 2011). The solvents were evaporated under reduced pressure at 40^{0}C using a rotary evaporator (Rotary Evaporator, Heidolf, Germany).

The extracts derived are referred as

- Hydro alcohol extract of *Eugenia jambolana (HAE-EZ),*
- Hydro alcohol Extract of *Cinnamomum Zeylenicum (HAE-CZ)* and
- Aqueous Extract of *Cinnamomum zeylenicum (AQE-CZ).*
- *HAE-EZ* & *HAE-CZ* (80:20);
- *HAE-EZ* & *AQE-CZ* (70:30);
- *HAE-CZ* & *AQE-CZ* (60:40) ;
- *HAE-EZ*; *HAE-CZ and AQE-CZ (60:30:10).*

The extracts were further used for evaluating its anti-diabetic potential.

INVITRO EVALAUTIONS

CELL CULTURE

Adipocytes Myocytes and Hepatocytes cell lines namely 3T3L1, C2C12 and HepG2 were procured from National Centre for Cell Sciences, Pune. The cells were maintained in laboratory conditions and prior to the experimentation they were grown in medium and maintained.

The 3T3L1 (mouse fibroblast) cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Calf Serum (FCS), 4mM Glutamine and 1% antibiotic/antimycotic, in a 5% CO_2 incubator at 37^{0}C. The C2C12 (myocytes) cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS), 4mM Glutamine and 1% antibiotic/antimycotic, in 5% CO_2 incubator at 37^{0}C. Whereas HepG2 cell lines were cultured in Eagle's minimal essential medium (EMEM) containing 10% FBS and 4mM Glutamine, in a 5% CO_2 incubator at 37^{0}C. All the cells were grown in a T-75 flask and experiments were performed at 90% confluence.

The 3T3L1 cells were seeded at a cell density of 6 X 10^4 cells/well at a final volume of 1000 µl in a 24 well plate with DMEM containing 10% FBS and incubated it for 48 hrs until the cells become confluent. C2C12 cells were seeded at a cell density of 3 X 10^4 cells/well at a final volume of 100 µl in a 96 well plate in DMEM containing 10% FBS and incubated it for 48 hrs. Simultaneously the HepG2 cell lines were seeded at a cell density of 1 X 10^4 cells/well at a final volume of 100 µl in a 96 well plate in EMEM containing 10% FBS and incubated it for 48 hrs for making the cells confluent.

CELL DIFFERENTATION AND PROCESSING

After 48 hrs of incubation the old media was removed in 3T3L1 cell lines and 10% FBS was added and incubated again for 2 more days. Whereas for C2C12 and HepG2 cell lines were differentiated with 5% FBS and incubated for 2 days respectively. The preadipocytes, which are undifferentiated fibroblasts, are induced to differentiate to mature adipocytes using specific MID differentiation stimulus (high concentrations of Isobutyl-methylxanthine (IBMX), insulin and dexamethasone) (Langer Safer *et al.,* 1985). The C2C12 myoblast and HepG2 hepatocytes are induced to differentiate to mature cells using specialized media (e.g. serum-free DMEM medium). Finally the differentiated adipocytes, myocytes and hepatocytes were tested for their extra-cellular glucose uptake activity in response to treatment with physiologically relevant concentrations of insulin.

MEASUREMENT OF GLUCOSE UPTAKE ACTIVITY

Concentrations ranging from 0.034µg to 33.4µg were tested for insulino mimetic and sensitization effects with or without insulin. The study was carried out using radio labeled insulin for measuring the changes in the level of glucose uptake activity of cells in response to treatment with samples (Yu-Chiao Yang *et al.,* 2003). The assay was performed in a 96-well micro titer plate and the counts per minute (CPM) were measured using a radioactive cell counter. The amount that showed best insulin mimetic and sensitization activity in 3T3L-1 C2C12 and HepG2 cells for glucose uptake was around 0.334µg. The method (Liu *et al.,* 2001; Vogel, 2008) was employed for screening the glucose uptake activity**.**

The preadipocytes, premyocytes, hepatocytes and cultured in DMEM containing 10%FCS, 4mM Glutamine, 2 % NaHCO3 and antimycotic, in an atmosphere of 5% CO_2 at 37^{0}C. Myoblasts are cultured up to 80% confluency and the cells are sub- cultured at three-day intervals. Approximately 2 x 10^4 cells were seeded separately in each well of a 96 well plate and differentiated for 48 hours in DMEM:F12(1:1), 0.5mM of IBMX, 0.25mM dexamethasone and 1µg Insulin for 48hrs followed by incubation with 1µg of Insulin for 5 hours.

The ability of the plant extract to induce glucose uptake was tested in two different ways i.e., glucose uptake in presence of insulin plus extract and in absence of insulin (extract alone). The samples were incubated in duplicate (one set to evaluate glucose uptake in presence of insulin i.e. extract + insulin and other set without insulin i.e. extract alone) with different concentration of extracts (300µg/well, 30µg/well, 3µg/well and 0.3µg/well) in triplicates for 18 hours at 37^0 C and 5%CO_2, l00µl of DMEM. The medium is then removed and the cells are incubated with Krebs Ringer Hepes (KRH) buffer 100 µl at 37^0C and 5% CO2 for 10 minutes. For standard insulin response cells were incubated with insulin (positive control) at concentrations of 5µM, 10µM, 25µM, 50µM and 100 µM in KRH buffer in triplicates. The cells were treated with five different concentrations of 0.1, 1, 10, 100 and 500 µg/ml of extracts in triplicates for 20 min at 37^0 C and 5%CO_2 for measuring insulin mimetic activity. Insulin with a concentration of 10 µM (showed maximum response).

Glucose uptake reaction was initiated by adding 0.5 mM tritiated 2-deoxy glucose (final concentration 0.0062 tBq/mM) i.e., 100 µl/well, in the same plate containing insulin/herbal extract and the plate was incubated for 15 mins at 37°C in a 5%CO_2 incubator. After incubation, ice-cold KRH buffer added to stop the reaction and the plate was kept for 2 minutes in the freezer. The cells were washed with ice-cold KRH buffer to this 40 µl of 1x tritonX100 was added for protein estimation or else 60 µl of KRH was added and the plate was kept at -20º C for overnight incubation.

The cells were scraped and transfered them to iso-black plate. Finally 160 µl of scintillation fluid was added and mixed properly. The scintillation vials were used for measuring radioactive counts. The glucose uptake measurement was made in triplicate for concordance. Results were expressed as % glucose uptake with respect to solvent control.

Cell Viability Assay

The cell viability studies were performed using MTT calorimetric assay (Mosmann and Tim 1983). MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-arboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) working solution (1ml of MTS solution + 50µl 0f PMS (phosphate-buffered saline) solution) was prepared. The extracts were added and kept for 20 minutes for incubation, then the medium was removed from the wells and 100µl of normal growth medium was added. MTS working solution of 20µl/well was added and incubated for 30min at 37^0 C and 5%CO_2 (Cory et al., 1991). The absorbance was recorded at 490 nm using UV visible spectrophotometer (LABINDIA Model 3000+). The highest concentration where percentage survival is at > 90% was used as the starting concentration in the glucose uptake assay. The final results are expressed interns of percent increase/decrease in insulin mimetic and insulin sensitization activity.

α-GLUCOSIDASE INHIBITION ASSAY

α-glucosidase Inhibitors which act as competitive inhibitors of intestinal α-glucosidase can delay the digestion and subsequent absorption of elevated blood glucose levels. The extracts were pre-incubated with the enzyme before adding the substrate p-nitrophenyl- α-d-glucopyranoside (PNPG). α -glucosidase activity was measured by determining the color developed by the release of p-nitrophenol arising from the hydrolysis of substrate PNPG by α –glucosidase using spectrophotometric method (Shim *et al.,* 2003; Halvorson, 1966).

The α glucosidase inhibitory activity was performed with a set of eppendorf tubes which was labeled as blank, controls and samples (different concentrations) and were arranged in the stand. To all the test tubes 600µl of potassium phosphate buffer, 100µl of test sample and 25µl of enzyme (α-glucosidase., 1.2 EU/ml) was added accordingly. The samples were vortexed and Incubated at 37 ^{0}C for 15 minutes, after incubation 25 µl of PNPG was added and kept again Incubated at 37 ^{0}C for 15 minutes. Finally the reaction was terminated by adding 750 µl of Na_2CO_3. For Blank the reagents was added in reverse order. The Control doesn't have any sample or test solution. A set of color control was done for the test samples without enzyme and PNPG but maintaining the volume with buffer. The absorbance of all the samples was measured at 405nm using UV visible spectrophotometer (LABINDIA 3000+). The percent inhibition of enzyme activity by the test sample was calculated as:

$$\% \text{ Inhibition} = \frac{AC_{405} - (AT_{405} - Cc)}{AC_{405}} \times 100$$

Where AC= Absorbance of Control read at 405nm
AT= Absorbance of Test Sample read at 405nm
Cc= Color Correction
Commercially available Acarbose was used as a standard and compared with all the extracts. The results were expressed in the form of IC_{50} value (mg/ml) i.e., the concentration of extract required to inhibit 50% of the enzyme activity at standard conditions.

INVITRO ANTI OXIDANT ASSAY

The antioxidant activity of the all the plant extracts and standard was assessed on the basis of the free radical scavenging effect of the stable DPPH (Badami *et al.,* 2003; Farrukh *et al.,* 2006). The plant extracts was dissolved in DMSO at a concentration of 3 mg/ml

(150 µg/ml in final reaction mixer) and was serially diluted in DMSO by two fold including the standard (ascorbic Acid) at a concentration of 1 mg/ml. Every time DPPH was prepared fresh in absolute alcohol at a concentration of 4.9mg/25ml. The mixture consisted of 125 µl of DPPH, 100 µl of freshly prepared 0.5mM Tris buffer (pH 7.2) and 25 µl test extracts/standard was prepared in a 96 well plate and incubated at room temperature for 10 min. The absorbance was measured at 517 nm by using UV-visible Spectrophotometer (LAB INDIA 3000+ Series). The results are expressed as percent ascorbic acid equivalents (AAE) for DPPH.

SATISTICAL ANALYSIS

The results were analyzed by using one-way ANOVA to establish the statistical significance. The least significant difference was used to determine significant differences between individual samples. Values were considered to be significantly different from the control if $p < 0.05$.

RESULTS

All observed values of glucose uptake activity are blank corrected using the control (cells alone background value). These values are normalized with MTT cell viability assay values for the corresponding extracts. The degree of insulin mimetic/sensitization activity of each sample concentration are calculated as percentage of that observed using standard insulin at 10nM alone (data not shown). Results of Insulin mimetic and insulin sensitization activity of extracts were shown in Table 1 and Table 2.

In 3T3-L1 cell lines the glucose uptake assays, a significant level of insulin mimetic activities were observed in the case of extract APKJ-004 (42%) and AQE-CZ (32.1%). While in the case of C2C12 based glucose uptake assays, majority of the APKJ extracts tested in this study displayed significant levels of insulin mimetic activity wherein the most pronounced insulin mimetic potential was shown by extract APKJ-004 (87.2%) followed by the other extracts in the following decreasing order: APKJ-001 (71%); HAE-CZ (69.4%); APKJ-002 (65%) APKJ-003 *(53.6%) and* HAE-EJ (48.5%) and AQE-CZ (41.3%).

Insulin sensitization activity, significant levels of activity (i.e. > 100% of insulin-alone treatment) were shown by all the seven APKJ extracts tested. Among the screened extracts APKJ-004 *(376.2%)* in 3t3 L1 cells and APKJ004 (423%) in C2C12 cell lines has showed highest sensitization potential to that of other extracts. In HepG2 Cell lines the significant insulin mimetic activity was observed in APKJ004 (89.5%) and Insulino sensitization activity was found to be APKJ004 (367%). The results have clearly indicated that the poly herbal extract APKJ004 has good potential to serve as an antidiabetic agent with insulin mimetic and sensitization activity was observed in APKJ-004 (i.e., C2C12 cell lines) extract when compared to others extracts.

The α-glucosidase inhibitory activity results were expressed in the terms of IC_{50} values. The most potent alpha-glucosidase activity was shown by extract APKJ-004 (IC_{50} of 147.2µg/ml) when compared to that of standard Acarbose (132.3 µg/ml). The results obtained are represented in Fig 1.

The antioxidant property was shown by all the extracts. Among the screened extracts APKJ-004 (36.17%) has shown prominent activity while others showed in the order of decreasing potential. The results were comparable to the standard ascorbic acid (Fig 2).

Table 1: Table depicting percent insulin sensitization activity at 3.34 µg concentrations of extracts compared to that of 10nm insulin in 3T3-L1, C2C12, and HepG2 cell lines glucose uptake assay.

Extract name	Percent Insulin Sensitization Activity at 3.34 µg concentration (Glucose Uptake)		
	3T3 L1 Cell line	C2C12 Cell line	HepG2 Cell line
10 nm Insulin Std.	185 ± 9.25	185 ± 9.25	205 ± 10.25
HAE-EJ + 10nm Insulin Std.	215 ± 10.75	217 ± 10.85	235 ± 11.75
HAE-CZ + 10nm Insulin Std.	273.2 ± 13.66	256 ± 12.8	215 ± 10.75
AQE-CZ + 10nm Insulin Std.	254.1 ± 12.70	261 ± 13.05	260 ± 13
APKJ001 + 10nm Insulin Std.	246.7 ± 14.67	223 ± 11.15	210 ± 10.5
APKJ002 + 10nm Insulin Std.	309.6 ± 15.48	285 ± 14.25	299 ± 14.95
APKJ003 + 10nm Insulin Std.	288.5 ± 16.1	190 ± 9.5	345 ± 17.25
APKJ004 + 10nm Insulin Std.	376.2±18.81	423 ± 21.15	367± 23.95

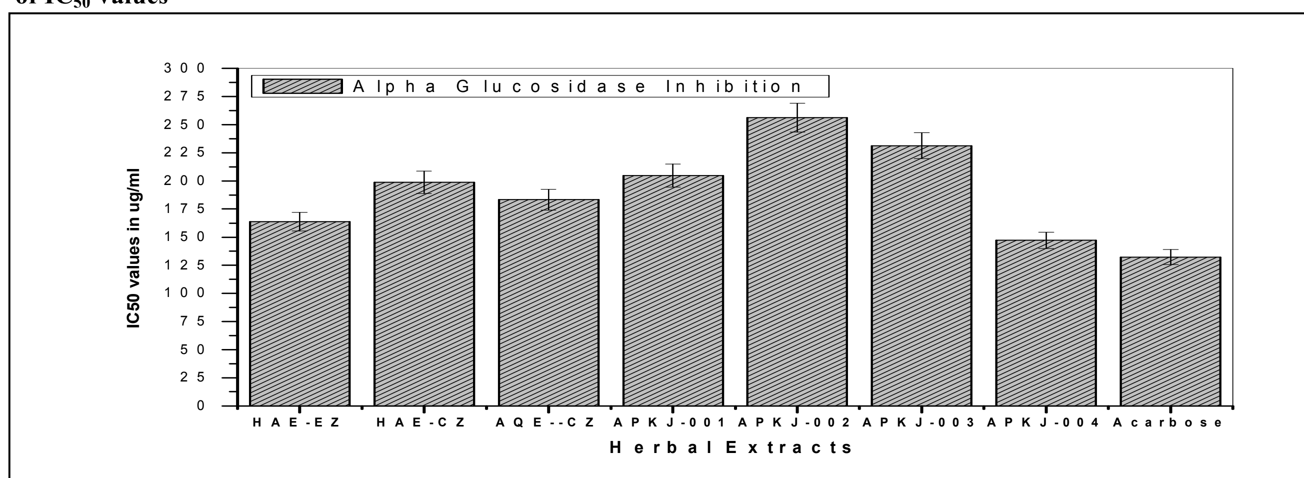
All the value represent (Mean ± S.E) derived from two experiments with 3 replicates. The results are statistically significant (p>0.05)

Table 2: Table depicting percent insulin mimetic activity at 3.34 µg concentrations of extracts compared to that of 10nm insulin in 3T3-L1, C2C12, and HepG2 cell lines glucose uptake assay.

Extract name	Percent Insulin Mimetic Activity at 3.34 µg concentration (Glucose Uptake)		
	3T3 L1 Cell line	C2C12 Cell line	HepG2 Cell line
10 nm Insulin Std.	100 ± 3.45	100 ± 5.32	100 ± 5.91
HAE-EJ	25.3 ± 3.25	48.5 ± 2.42	62.3 ± 3.11
HAE-CZ	29.8 ± 1.45	69.4 ± 3.47	74.8 ± 3.74
AQE-CZ	32.1 ± 1.15	41.3 ± 2.06	69.3 ± 3.46
APKJ001	21 ± 1.05	71 ± 3.55	79.7 ± 3.98
APKJ002	31 ± 2.15	65 ± 3.25	63.1 ± 3.15
APKJ003	26.3 ± 1.3	53.6 ± 2.68	80.4 ± 4.02
APKJ004	42 ± 1.64	87.2 ± 4.36	89.5 ± 4.47

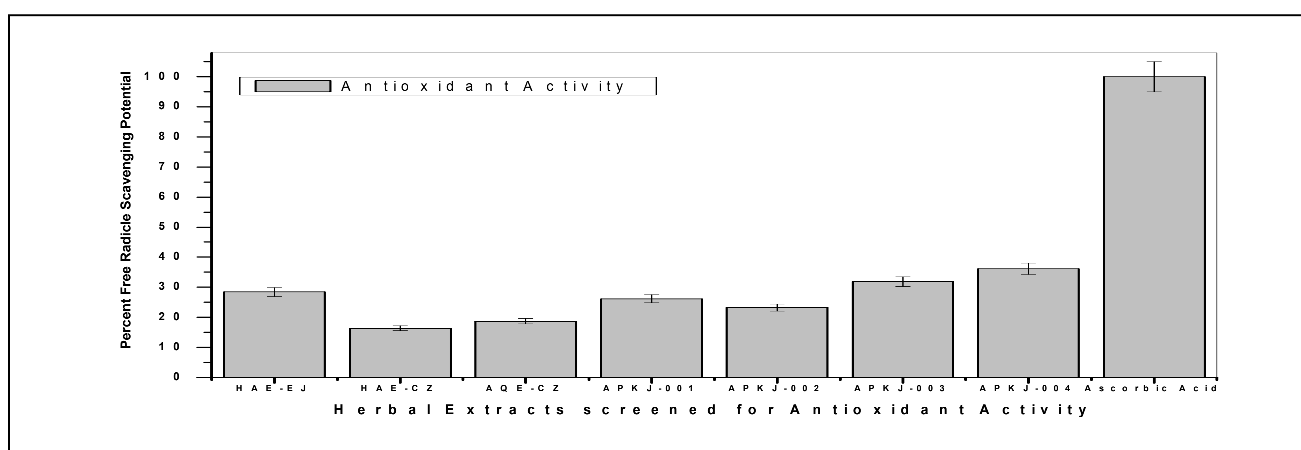
All the value represent (Mean ± S.E) derived from two experiments with 3 replicates. The results are statistically significant (p>0.05)

Figure 1: Charecteristics of Inhibitory potential of the extracts evaluated for its alpha glucosidase activity in terms of IC_{50} values



All the value represent (Mean ± S.E) derived from two experiments with 3 replicates

Figure 2: Antioxidant activity of herbal extracts in comparison with standard ascorbic acid



The values represented (Mean ± S.E) are derived from two experiments with 3 replicates

DISCUSSION

Diabetes mellitus is a chronic disease characterized by increased blood glucose levels and disturbances in the carbohydrate, fat and protein metabolism (Apparao *et al*., 2003). The insulin stimulated glucose uptake in adipose tissue and skeletal muscles is critical for reducing post pranadial blood glucose concentration. Dysregulation of this process is one of the important factors in type 2 diabetes. Many oral pharmacotherapies for the management of diabetes mellitus have emerged out with this interest and are widely used till today. The therapy may act by mimicking insulin or either by stimulating insulin release or by potentiating insulin action or by reducing hepatic glucose production.

Earlier for identification of lead molecules (Venter *et al.,* 2008) proposed that using single cell line/metabolic pathway/ enzyme activity may fail to identify the possibility of lead molecule. To avoid such ambiguity in identification of lead molecules the present study was designed with three cell lines. The study has shown good activity and the results were comparable to the standard. The insulin mimetic and sensitization activity of APKJ 004 was prominent in skeletal cell lines (252.4%) when compared to adipose and liver cell lines. Skeletal muscle accounts for more than 80% of the total insulin mediated glucose uptake (Kumar and Dey, 2003) and the results obtained in our study are comparable.

The inhibition of α-glucosidase enzyme activity is one of the mechanism through which the polyherbal extract exerts its hypoglycemic effect. Results clearly demonstrated that the polyherbal extract APKJ-004 has a significant inhibitory activity at IC_{50} 147.2µg/ml and is comparable to the standard acarbose. Therefore the extract APKJ-004 may play important role in the development of neutraceuticals and also in the management of diabetes.

The oxidative free radicals have been implicated in the pathogenesis of diabetes mellitus. In addition the diabetic patients have significant defects of antioxidant protection. It is believed that the metabolic disorders in diabetes mellitus may be due to the enhanced cellular oxidative stress and reduced antioxidant potential. Results in our study depicted that the polyherbal extract APKJ -004 (30%) of free radical scavenging activity equivalent to that of the standard. The study revealed a very potent antioxidant indicating its role in aiding to the pharmacotherapy of diabetes.

The animal models are more appropriate for screening antidiabetic activity. Study reported with Invitro evaluations warrants for further assessment by using invivo techniques which will help in understanding the mechanism of action. Study in this direction is under process.

CONCLUSION

The present study has shown very encouraging results for the treatment of diabetis mellitus in *invitro* evaluations. The statement is authenticated with cell culture studies, α-glucosidase and antioxidant properties. Based on the lead obtained from invitro studies, plan an *invivo* experiments for further confirmation of the obtained results.

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