



## Preclinical Evaluation Of Polyherbal Formulations: Hypoglycemic Activity In Rats.

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*Diabetes mellitus is a metabolic disorder and the disease management is an important measure for the pharmacotherapy. The medicinal plants play very vital role in preventing the advancement of the disease. The polyherbal extract was prepared from Eugenia Jambolana-seed and Cinnamomum zeylenicum-bark as hydro alcohol and aqueous extracts. The extracts were screened for invitro antidiabetic activity. Among all screened extracts the polyherbal extract APKJ-004 showed prominent invitro antidiabetic activity. The present study was designed for assessment of toxicity and therapeutic efficacy of the selected polyherbal extract APKJ-004. The acute and sub acute toxicity were conducted in wistar rats. The results of toxicity assessment revealed that clinical, biochemical and Histopathological parameters studied were in normal range and comparable to controls. The study revealed that no toxic symptoms observed throughout the period of exposure. The invivo evaluation results showed that the extract APKJ-004 reduced the elevated glucose levels ( $97.01 \pm 6.23$ mg/dl) were comparable to the glibenclamide ( $89.3 \pm 7.42$ mg/dl). The insulin levels were considerably increased in polyherbal treated group from  $06.75 \pm 0.96$   $\mu$ U/ml to  $23.13 \pm 0.35$   $\mu$ U/ml. The glucose tolerance test results revealed the absorption efficiency of APKJ 004 was found to be  $112.3 \pm 6.68$  mg/dl when compared with standard glibenclamide ( $93.4 \pm 4.67$ mg/dl) treated group at 120 min. Based on the above facts it is concluded that APKJ-004 polyherbal extract act as a potent antidiabetic agent with minimal or no side effects and useful in the pharmacotherapy of diabetes.*

**Key words:** Hypoglycemic Activity, polyherbal extract, Diabetes Mellitus, Streptozotocin Preclinical toxicity.

### INTRODUCTION

Diabetes mellitus is a multifaceted metabolic disorder characterized by elevated blood glucose levels which results from deficiency in insulin secretion, insulin action and or both<sup>1</sup>. The world wide increasing incidences on diabetes mellitus constitute a global public health problem reaching epidemic proportions<sup>2</sup>.

Diabetes mellitus is considered to be a serious issue in many countries and traditional methods using medicinal plants to control diabetes is gaining momentum<sup>3</sup>. The allopathic medicine for control and management of diabetes without adverse effects is still a challenge for many researchers<sup>4</sup>. The synthetic hypoglycemic agent does produce serious side effects<sup>5</sup> and whereas drug derived from medicinal plants are frequently consider being safe & cost effective. To avoid the side effects and effective treatment warrants the use of natural products<sup>6</sup>.

Therefore the present study has been designed to evaluate the herbal preparations used to treat diabetes, as an alternative therapy but their reported hypoglycemic effects are multifarious. The Literature survey revealed that several NCE's showed the antidiabetic activity but the study lacks the proper scientific validation and systematic evaluation<sup>7</sup>. Herbal preparations provide a useful source of new oral hypoglycemic agents for development as pharmaceutical entities or as simple dietary adjuvant to existing

therapies. The polyherbal extract have a proven efficacy compared to single plant extract<sup>8</sup>. Based on the *invitro* evaluations the identified formulation was subjected for preclinical toxicity studies. The present study has been designed to evaluate herbal preparations with suitable animal model to authenticate the *invitro* evaluations<sup>9,10</sup>. The extracts were subjected for acute, sub acute toxicity studies and evaluated as a hypoglycemic polyherbal preparation in animal model for determining its safety and efficacy.

## MATERIALS AND METHODS

**Materials:** Streptozotocin (STZ) was purchased from Sigma, St Louis, MO, USA. Glibenclamide was gift from Bright labs, Hyderabad. Biochemical kits and all other chemical used were of analytical grade.

### Plant Extract:

The plant materials *Eugenia jambolana* and *Cinnamomum zeylanicum* were collected from Acharya N.G Ranga University and taxonomic identification of the specimen was authenticated by Dr.P.Jayaraman, taxonomist at Plant Research Centre, Chennai and a voucher specimen wide Regd. No's PARC/2010/893, PARC/2010/894 is preserved. The polyherbal extract APKJ 004 was prepared by using hydro alcohol extract of EZ, hydro alcohol extract of CZ and aqueous extract of CZ. The extracts were extracted with ethyl alcohol (95%) and water at temperature (65-75<sup>0</sup>C) using direct soxhlet method<sup>10</sup>. The solvents were evaporated under vacuum and reduced pressure at 40<sup>0</sup>C using a rotary evaporator (HEI VAP Advantage HI/HB/G3, Heidolf, Germany). Based on the *invitro* evaluations of single and combinations of the extracts the extract APKJ-004 (HAE-EZ; HAE-CZ and AQE-CZ (6:3:1)) has shown prominent hypoglycemic activity was further selected for studying efficacy and safety parameters for measuring its significance in the treatment of diabetes<sup>9</sup>.

### Experimental rats:

Albino Wistar rats weighing between 160-200 g were procured from National Institute of Nutrition, Hyderabad, India. The animals were housed as per CPCSEA guidelines i.e., six animals per each polypropylene cage under standard conditions of temperature (22±3<sup>0</sup>C) and relative humidity (30-70%) with a 12:12 light: dark cycle under controlled environment (Regd.No. 1412/a/11/CPCSEA). The animals were fed with standard pellet diet (Mahaveer Enterprises feeds Ltd., Bangalore.) and water *ad libitum*. The animals were fasted for 18 hours before commencing the experiment. The study was approved by Institutional Animal Ethics Committee (IAEC) and designated by study code SOP/PS/IAEC/006.

### HPTLC analysis of the extract:

HPTLC Finger print analysis of *Eugenia jambolana* and *Cinnamomum zeylanicum* and their combination as polyherbal extracts were performed on 10X10 cm HPTLC plates coated with 0.5mm layer of silica gel 60F<sub>254</sub> (Merck, Darmstadt, Germany). Before using, the plates were washed with methanol and activated at 110 degree centigrade for 5 min. Samples were applied as 4mm wide bands and 6mm apart by using a Camag (Muttenez, Switzerland) Linomat IV sample applicator equipped with a 100ul syringe. A constant application rate of 6ul/sec was used. Mobile phase was Methanol:Chloroform:GAA (Glacial acetic acid)

(70:30:0.1), reducing agent vanillin H<sub>2</sub>SO<sub>4</sub> and chromatograms were monitored at 600nm on HPTLC system<sup>12</sup>.

### **Acute toxicity studies:**

The acute toxicity study was carried out by guidelines set by OECD 420 guidelines. Wistar rats (160-200g) maintained under standard laboratory condition was used. A total number of six animals were used per group which received a single dose (2000 and 5000mg/kg, b.wt (p.o.) of polyherbal drug. Animals were kept overnight fasting prior to drug administration. After the administration of polyherbal drug, the food was withheld for 3-4 hours. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24h (with special attention during the first 4 hours) and daily thereafter for a period of 14 days. Daily cage side observation included changes in skin and fur, eyes and mucous membrane (nasal) and also respiratory rate, circulatory, autonomic changes was observed<sup>13</sup>.

### **Glucose Tolerance Test (GTT)**

Animals were fasted 18 hrs<sup>14</sup> with free access to water and were separated in 5 groups of 6 rats each. Animals of all groups were administered with oral D-glucose solution 2 gm/kg using oral gavages, Group second, third and fourth were treated orally with polyherbal extract at a dose of 200, 400 and 800 mg/kg b.wt., (p.o.) each for 30 min before the oral administration of glucose load. Control animals were treated with vehicle, blood sample were withdrawn from the retro-orbital plexus region of each animals under ether anesthesia at 0 min, 30 min, 90 min and 120 min after glucose challenge and the fifth group received glibenclamide (2 mg/kg as positive control). Blood glucose levels were estimated using GOD-POD kit (BioEra Life Sciences Pvt. Ltd., Pune).

### **Streptozotocin induced Diabetes:**

Diabetes mellitus was induced to rats at a dose of 45 mg/kg/ body weight i.e., 5 ml of STZ (sigma chemical Co. USA) by intraperitoneal (i.p.) injection dissolved in 0.1M citrate buffer, and equal volume of buffer was dosed in normal<sup>15</sup>. After forty eight hours of STZ administration, blood samples were withdrawn from retro orbital plexus and glucose levels were determined to confirm induction of diabetes. The rats exhibiting >250 mg/dl were confirmed to be diabetic and they were selected for further studies. The grouping of were done accordingly Normal rats were injected with physiological saline as Group 1, Diabetic control were wistar rats receiving 0.5% CMC as vehicle as Group 2, diabetic rats receiving polyherbal extract (APKJ-004) 200 mg/kg body weight as Group 3, Diabetic rats receiving polyherbal extract (APKJ-004) 400 mg/kg body weight as Group 4, Diabetic rats receiving polyherbal extract (APKJ-004) 800 mg/kg body weight as Group 5 and finally Diabetic rats receiving 2mg/kg Glibenclamide drug as Group 6. After blood collection, animals were culled using ether anesthesia and the vital organs were excised and subjected to further biochemical analysis.

### **Biochemical and Hematological analysis**

Blood glucose was measured by using glucose-oxidase method<sup>16</sup> Insulin was assayed by radio-immunoassay kit method (BioEra Life Sciences Pvt. Ltd., Pune). Biochemical and hematological analysis were carried out using Semi Auto Bio-Chemistry Analyzer (Optima S, LABINDIA) and hematological analyzer (PE- 6800 VET, Procan Electronics) on termination day. The effect of polyherbal extract APKJ-004 on vital organs such as kidney, liver, pancreas, heart etc., were determined on day 29. The body weight and food intake were monitored and recorded using animal weighing balance (Schimadzu, UXW-4200H) during 0, 7, 14, 21 and 28 days.

## Histopathological analysis:

On the termination day of the study the animals were sacrificed and dissected the required organs such as liver, pancreas, kidney, and heart and they were fixed in 10% formalin. The sections of the tissues of 5-6 $\mu$ m were cut and stained with haemotoxylin and eosin stains. The tissue sections were subjected to rehydration by exposing them to decreasing concentration of alcohol i.e., 100-30%. Then the sections were stained with haemotoxylin. Later the sections were dehydrated by using increasing concentrations of alcohol and stained with eosin. Finally the sections were treated with DPX (Diphenylxylene) and mounted on to the slide for examination under microscope<sup>17</sup>.

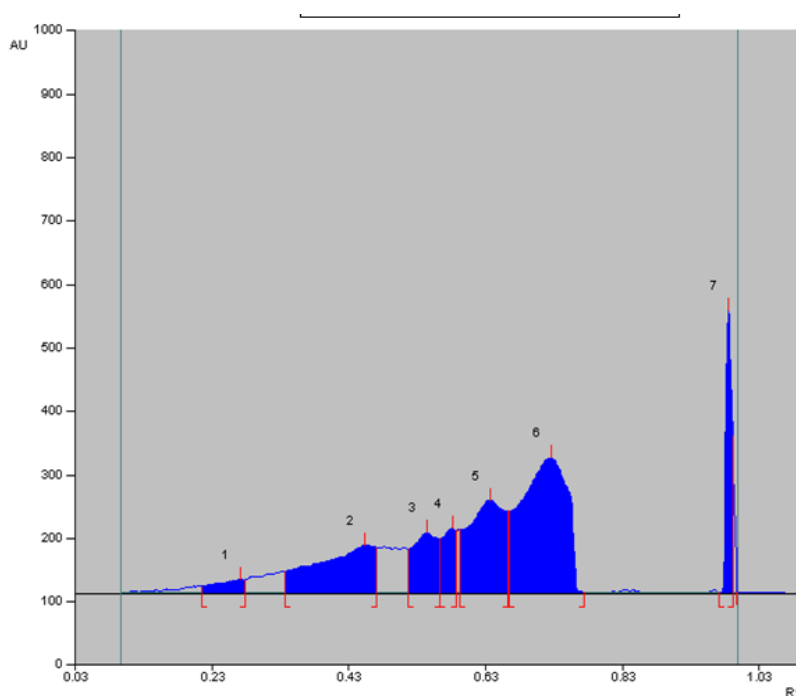
## Statistical analysis

All the data are reported as Mean $\pm$ S.E. The results were statistically analyzed by one way analysis of variance (ANOVA) by Origin 7.6 software , followed by Duncans multiple range test (DMRT). P<0.05 was considered to be significant.

## RESULTS

### HPTLC finger printing analysis:

Preliminary phytochemical analysis of APKJ -004 revealed presence of phenolic compounds, flavonoids, glycosides, alkaloids, carbohydrates, terpenoids and anthocyanins. HPTLC analysis indicated that the presence of seven distinct peaks. The densitogram of HPTLC analysis on APKJ-004 were depicted in the Figure-1



**FIGURE 1: Densitogram of polyherbal Extract- APKJ-004 determined by using CAMAG HPTLC analysis system**

**Acute toxicity study:**

The lethal dose 50 was determined in rats and none of the animals died at the dose of 2000mg/kg b.wt, and animals were survived at 5000 mg/kg b.wt. The LD<sub>50</sub> of the polyherbal extract was found to be more than 5000 mg/kg. During the observation period of 14 days, control and treated groups body weight, biochemical and hematological parameters were similar. There was no statistical difference when compared with controls. None of the animals died during the study and no abnormal behavior was observed in treated group.

**Sub acute toxicity Study:**

Sub acute toxicity studies on polyherbal extract APKJ-004 treated at 200,400 and 800 mg/kg b.wt., on rats for 28 days did not showed any abnormal changes in biochemical, hematological studies. During the observation period the body weight increased throughout the exposure period when compared to that of control (initial 189.12±.18.3 and termination day 314.53±26.13, 171.42±10.30 on initial day to termination day 245.14±12.34 for male and female rats respectively). The results of the body weight of male and females rats were represented Figure-2. The absolute and relative organ weights were determined on termination day and were expressed as 100gm/wt. The weights measured on vital organs at the termination day did not effect the organs and they are comparable to that of controls. The biochemical and hematological results were in the normal range. The histopathological studies were subjected to pathological evaluation. The evaluations revealed no abnormal changes in the vital organs studied. Histopathological slides were preserved

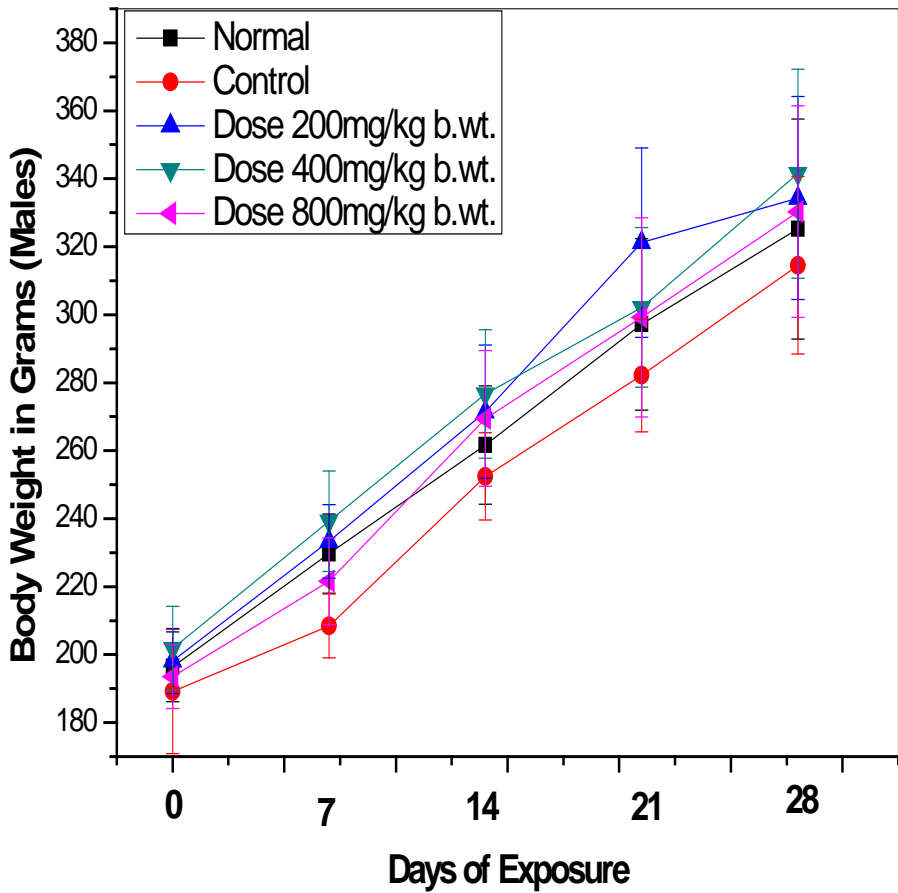
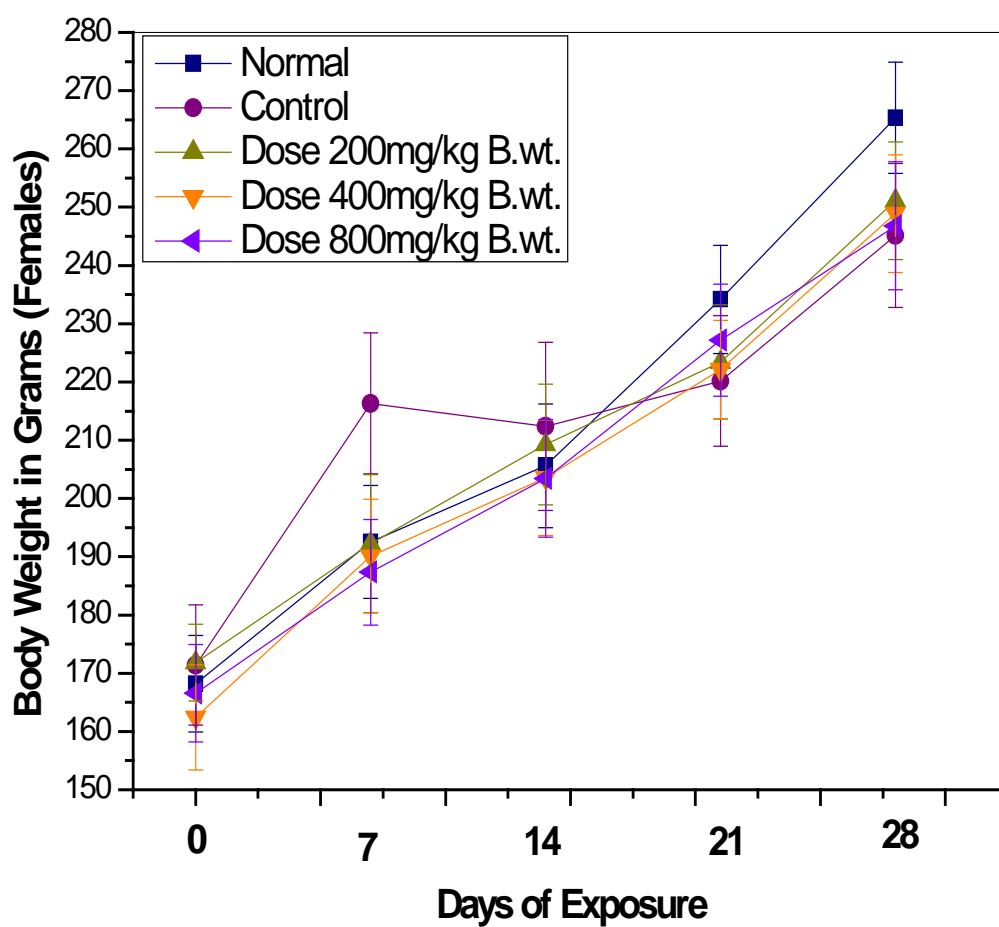


Figure: 2 (a)



2(b)

STZ Induced Diabetes:

The blood glucose levels were increased from  $80.5 \pm 6.75$  to  $268.01 \pm 31.14$  mg/dl in control group. The treated group decreased the blood glucose level significantly  $p > 0.05$ . In case of group 5 the blood glucose level was lowered significantly when compared with the control diabetic group at a dose of 800mg/kg b.wt ( $97.01 \pm 6.23$  mg/dl). The blood glucose levels were lowered in all the treated groups when compared to the diabetic control group in dose dependent manner. The glibenclamide treated group lowered the blood glucose level to the extent of  $89.3 \pm 7.42$  mg/dl.

### Plasma insulin

The plasma insulin estimated in control group was of  $6.75 \pm 0.96$   $\mu$ U/ml. The plasma insulin was significantly increased in group V  $23.13 \pm 0.35$   $\mu$ U/ml when compared to that of the control group ( $p < 0.05$ ). The results of group V and group VI with glibenclamide are comparable and there was no difference in insulin release significantly ( $p = ns$ ). Group 2 treated with STZ showed significant decrease in plasma insulin level and is highly significant when compared to control and normal ( $p < 0.001$ ). The results were tabulated in Table 1.

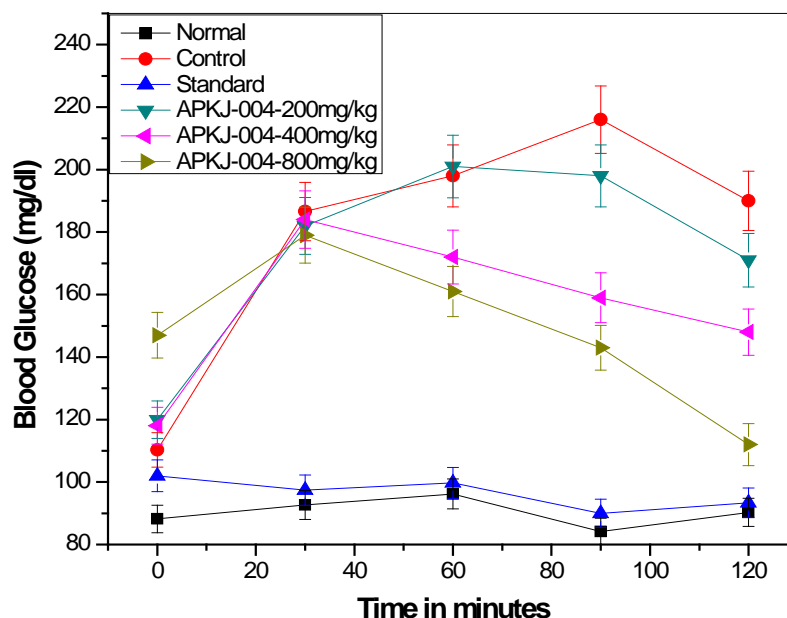
TABLE 1: Effect of polyherbal herbal formulation (APKJ-004) on blood glucose, insulin levels in diabetic rats.

Groups	Description	Blood Glucose (mg/dl)	Insulin ( $\mu$ U/ml)
I	Normal	$80.5 \pm 6.75$	$14.59 \pm 1.20$
II	Diabetic control	$268.01 \pm 31.14$	$06.75 \pm 0.96$
III	Diabetic + APKJ-004 (200mg/kg)	$143.33 \pm 5.45$	$11.31 \pm 0.87$
IV	Diabetic + APKJ-004 (400mg/kg)	$128.4 \pm 6.67$	$18.34 \pm 0.45$
V	Diabetic + APKJ-004 (800mg/kg)	$97.01 \pm 6.23$	$23.13 \pm 0.35$
VI	Diabetic + Glibenclamide(2mg/kg)	$89.3 \pm 7.42$	$19.02 \pm 0.82$

Data expressed as mean  $\pm$  standard error ( $n=6$  rats). The values of blood glucose were significant at  $p < 0.05$ , the values of insulin were significant at  $p < 0.004$ .

### GTT (Glucose Tolerance Test):

Rats were subjected for the glucose tolerance test. The blood glucose levels were significantly increased in control group. There was significant decrease in the elevated blood glucose levels at 200, 400 and 800 mg/kg ( $171 \pm 8.55$ ,  $148 \pm 7.47$  and  $112 \pm 6.70$  mg/dl) when compare to that of control ( $190 \pm 9.51$  mg/dl). The decrease in blood glucose levels was dose dependent manner. The results were encouraging when the polyherbal extract were subjected to the glucose tolerance test (Figure 3).



**FIGURE 3: Effect of polyherbal extract APKJ-004 on glucose tolerance test. Values are Mean±S.E, (n=6) and P values are significant at p<0.05.**

## DISCUSSION:

Diabetes mellitus is a chronic disease characterized by increased blood glucose levels and disturbances in the carbohydrate, fat and protein metabolism<sup>18</sup>. The preliminary phytochemical analysis revealed presence of phenolic compounds, tannins, flavonoids, glycosides, alkaloids and anthocyanins. Similar kinds of constituents were earlier reported<sup>19</sup>. Future study warrants for the identification, characterization and purification of the active components from polyherbal extract will give the clue for the lead molecules and chemical modification may yield good new chemical entity for the treatment of diabetes mellitus<sup>20</sup>.

It is known that the efficacy and toxicity are important for assessing the therapeutic uses of the polyherbal extract. Hence the acute and sub acute studies were carried out as per OECD guidelines and the results indicated that the polyherbal formulation used in the study do not have any toxicity related to behavior, clinical and biochemical parameters. The increase in body weight and food intake is indicated the



healthiness of the control and treated animals. The LD<sub>50</sub> values were >5000mg/kg b.wt. Hence it is classified as GHS (Global Harmonization System) category 5 as per the OECD guidelines.

To determine the therapeutic efficacy of the polyherbal extract in the treatment of diabetes mellitus, the methods were chosen as described by Vogel. The study with respect to glucose tolerance test showed significant increase in the test carried out by oral route<sup>21</sup>. The GTT reveals the influence of high blood glucose concentration and reveals the drug which affects the absorption of glucose. Taking this clue it was observed that glucose levels were significantly decreased indicating the therapeutic usefulness of polyherbal extract APKJ-004 in the treatment of diabetes mellitus. The results were comparable with known antidiabetic drug glibenclamide.

The streptozotocin induced diabetic animal showed significantly higher glucose levels when compared with normoglycemic agents<sup>22,23</sup>. The hyperglycemic activity in the control group was sustained but in polyherbal treated and glibenclamide decreased significantly. This indicated the polyherbal extract evaluated has similar action to that of glibenclamide. To prove the fact, the insulin release studies were done with control, polyherbal extract and glibenclamide. The insulin release was higher when compare to the control streptozotocin induced group of animals. The results indicated that the glibenclamide and polyherbal extract act in similar manner. The initial approach has revealed that the formulation APLKJ-004 has shown reasonably good activity as an antidiabetic drug. To determine the mechanism of action of polyherbal preparation are required to explore at the cellular level to have more direct evidence for the antidiabetic activity<sup>24,25</sup>.

## CONCLUSIONS:

Based on above studies it is concluded that the polyherbal APKJ 004 has many evidences to identify it as an oral hypoglycemic agent. Further a study at molecular level on the mechanism of this drug can give us a clear insight on its mode of action that makes it more effective than the commercially available standard drugs. The studies in these lines are in progress.

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