# PHARMACOLOGICAL EVALUATION OF *VIGNAMUNGO* SUPERCRITICAL CARBON-DIOXIDE FLUID EXTRACT IN STREPTOZOTOCIN INDUCED DIABETIC RATS FOR DIABETIC NEUROPATHY

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**Abstract**
A study was designed to determine preclinical activity of Vigna mungo seed Supercritical carbon-dioxide fluid extract and normal ethanolic extract in streptozotocin induced diabetic rats for diabetic neuropathy. The preliminary phytochemical analysis of Vigna mungo Supercritical carbon-dioxide fluid extract and normal ethanolic extract was performed by standard phytochemical procedures whereas preclinical evaluation for diabetic neuropathy activity was carried out in Streptozotocin induced diabetic rats. Materials and methods: Diabetes was induced in male Sprague Dawley rats with streptozotocin(60 mg/kg i.p) and were divided into seven groups namely Normal control, Negative Control, third and fourth test groups of Supercritical fluid extract seeds of Vigna mungo (250 mg/kg and 500 mg/kg b.w.) fifth group Standard drug Glibenclamide 5mg/kg i.p. +Metformin 25 mg/kg i.p. sixth and seventh test groups of ethanolic extract seeds of Vigna mungo (250 mg/kg and 500 mg/kg b.w.) After the 4$^{th}$ week of diabetes, induction treatment was started for further 28 days (5$^{th}$ to 8$^{th}$ week) with Results: Significant activity against diabetic neuropathy was shown by Supercritical fluid extract seeds of Vigna mungo. Discussion: Vigna mungo Supercritical carbon-dioxide fluid extract possess phytoconstituents with antidiabetic potentiality. Also, it offers a scientific platform and baseline information for further analysis such as isolation and standardization of its bioactive compounds as herbal alternative for diabetic complications.

**Keywords:** Supercritical fluid extract, Diabetic Neuropathy, Vigna Mungo

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## Introduction

Diabetic neuropathy (DN) is an important complication of diabetes mellitus resulting in a great deal of morbidity. The prevalence of diabetic neuropathy is about 26.1% in Indian population[1]. Diabetes and the associated complications has become a global epidemic disease of 21st century thereby affecting a greater fraction of population worldwide. Based on the current survey of International Diabetes Federation (IDF), there are almost 73.12 million people in India only who are suffering from Diabetes and globally it is expected to rise from 450 million in 2017 to 691 million by 2045[2]. Diabetes mellitus is a metabolic disease which damage different bodily organs, causing kidney failure, vision loss, autonomic and peripheral neuropathy, peripheral vascular disease, myocardial infarction, and cerebrovascular disease with stroke. Diabetes affects the central nervous system and produce disturbances such as behavioral changes, autonomic dysfunctions, altered neuro endocrine functions, and neurotransmitter alterations and thus leading to end organ damage[3, 4]. Various pathways involved in the pathogenesis of diabetic neuropathy and degeneration are polyol, hexosamine, protein kinase C, advanced glycation, poly (ADP-ribose) polymerase, oxidative stress, and inflammation. Oxidative stress and inflammation play a crucial role in the development and progression of late-stage complications of diabetes[5]. Diabetic neuropathy (DN) is an important complication of diabetes mellitus resulting in a great deal of morbidity. The prevalence of diabetic neuropathy is about 26.1% in Indian population. Peripheral diabetic neuropathy is characterised by symptoms such as tingling and numbness, sharp pains or insensitivity to pain, motor incoordination, loss of sense of vibration etc. Untreated, it may lead to loss of reflexes and deformities that may progress to gangrene. The condition is characterised by peripheral demyelination, decrease in the nerve conduction and degeneration of myelinated and demyelinated sensory nerve fibres[6]. There is no definitive treatment for DN at present. Tricyclic antidepressants, SNRIs, anticonvulsants, opioids and topical capsaicin have been tried in the management of painful neuropathy of which duloxetine and pregabalin have been approved by the US FDA[7].

*Vigna mungo* Linn. (VM) is commonly known as black gram and is mainly cultivated in India and Pakistan. Traditionally, VM is mentioned for its beneficial effects in many of the ailments like abscess, inflammation, rheumatism and asthma. Moreover, the seeds are also diuretic, emollient, appetizer, thermogenic, nervine tonic, laxative, aphrodisiac, astringent, styptic and galactagogue[8]. Thus, medicinal plants have an ever-increasing role to play in the treatment or management of many diseases, especially in developing countries where resources are limited, and the plants are readily available. It is well known that choosing the proper extraction method is necessary for the improvement of extract quality and yield. Supercritical fluid extraction is an efficient and environmentally friendly method to extract non-polar constituents from plant sources; especially for those thermal unstable compounds[9]. $CO_2$ is preferred as a solvent especially in the areas of functional food and pharmacological products because of its moderate supercritical points. Therefore, in the present study, supercritical $CO_2$ extraction was employed to prepare bioactive extracts from *Vigna mungo* Linn. (VM) is commonly known as black gram[10]. The novelty of the present manuscript resides in the fact that Pharmacological evaluation for diabetic neuropathy of *Vigna mungo* Supercritical carbon-dioxide fluid extract in Streptozotocin induced diabetic rats

## Materials and methods

### Identification and authentication of plant material

The dried seeds of the plant *V. mungo* were purchased from the local market (Rajgurunagar. Tal. khed Pune). The plant of *Vigna mungo* (L.) Hepper (Family: Fabaceae) herbarium was identified and authenticated by Botanical Survey of India, Pune.

### Preparation of Supercritical carbon-dioxide fluid Extract:

3000 gm of *Vigna mungo* L. Hepper seed powder prepared. Supercritical fluid extract prepared by Shourya supercritical, Sangali. The conditions maintained during the preparation of the super critical fluid extract of *Vigna mungo* seeds were as follows: *Pressure: 250 lbs, Flow rate for CO2: 2.0 mL, Flow rate for Ethanol: 02 mL, Temperature: 40$^oC$*

### Preparation of ethanolic Extract:

The dried seeds of the plant *V. mungo* were isolated, chopped into small pieces, dried in the shade at room temperature for seven days and powdered. The powder was extracted with ethyl alcohol by maceration to get a yield of 14.1% w/w of the ethanolic seed extract.

**Characterization of plant e*xtract***

Qualitative phytochemical analysis of the supercritical fluid extract and ethanolic extract of *Vigna mungo* seeds were carried out to determine the presence of phytochemicals which includes carbohydrates, protein, flavonoids, terpenoids, steroids, phenolic compounds, and glycosides[11]. Carbohydrate, Protein, Alkaloids, Flavonoids, Terpenoids, Steroids, Phenols and Glycosides were found present in the both supercritical fluid extract and ethanolic extract of *Vigna mungo* seeds.

**IAEC protocol approval and procurement of animals**

In the present study, male Sprague Dawley rats, weighing about 200 -250 gm body weight were used in the present study. Animals were obtained from the National Institute of Biosciences, Pune, NIB is registered with CPCSEA having registration number 1091/abc/07/CPCSEA and acclimatized to the laboratory conditions for 2 weeks.

Healthy adult male Sprague Dawley rats between 2 and 3 months of age and weighing about 200 – 250 g were used for the study. The animals were housed in polypropylene cages, maintained under standard conditions (12 h light: 12 h dark cycle; 25 &177; 30˚C; 35-60% humidity). They were fed with standard rat pellet diet (Nutrivet Life Sciences, Pune) and water ad libitum. The Institutional Animal Ethical Committee of Dr DY Patil College of Pharmacy, Akurdi, Pune, Maharashtra (**1554/ PO/Re/S/11/CPCSEA) (DYP COP/IAEC/ 2020/ 02)** approved the study.

**Acute oral toxicity testing**

The acute toxicity study of the supercritical fluid extract of *Vigna mungo* seeds was evaluated in rats using Organization for Economic Co-operation and Development guideline 420[12].

Normal healthy rats were divided into five groups of six animals each. Different doses (100, 250, 500, 750, 1000 and 2000 mg/kg body weight) of the supercritical fluid extract of *Vigna mungo* seeds were administered orally. The rats were observed continuously for 2 h for behavioral, neurological, and autonomic profiles and after 24 and 72 h for any lethality.

Normal healthy rats were divided into five groups of six animals each. Different doses (100, 250, 500, 750, 1000 and 2000 mg/kg body weight) of normal extract of *Vigna mungo* seeds were administered orally. The rats were observed continuously for 2 h for behavioral, neurological, and autonomic profiles and after 24 and 72 h for any lethality.

The acute toxicity study of the supercritical fluid extract of *Vigna mungo* seeds was evaluated in rats using Organization for Economic Co-operation and Development guideline 420[12].

**Pharmacological studies**

**Evaluation of effect of repeated dose treatment of supercritical carbon-dioxide fluid extract on STZ- induced early diabetic neuropathy using models thermal hyperalgesia and cold allodynia**

The animals will be divided into following groups with 6 animals in each group-

Group I: Normal Control, Negative Control,

Group II: Negative Control (Diabetic neuropathy (DN)

Group III: (DN) + Supercritical fluid extract obtained from the seeds of ***vigna mungo*** plant (dose 250mg/kg, p.o)

Group IV: (DN) + Supercritical fluid extract obtained from the seeds of ***vigna mungo*** (dose 500mg/kg, p.o)

Group V: Standard drug Glibenclamide 5mg/kg i.p. +metformin 25 mg/kg i.p.

Group VI: (DN) + Normal Ethanolic extract of seeds of ***vigna mungo*** plant (dose 250mg/kg, p.o)

Group VII: (DN) + Normal Ethanolic extract of seeds of ***vigna mungo*** plant (dose 500mg/kg, p.o)

Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of fresh STZ (60 mg/kg body weight) in citrate buffer (0.1M, pH 7.4). After 48 h of STZ injection, fasting blood samples were withdrawn from tail vein and blood glucose level was measured by use of a glucometer (Accu-Chek, Johnson and Johnson, India). The animals having fasting blood glucose level ≥230 mg/dl were randomized in groups. The development of DNN was confirmed by basal nociceptive reaction at the 4$^{th}$ week of STZ injection, and all treatments were started thereafter from 5$^{th}$ to 8$^{th}$ week. At the end of 8$^{th}$ week, behavioral analysis were done and the blood sample was collected, No mortality was observed during the study period.

**Behavioral Markers**

**Locomotion activity**

Photoactometer test was performed to study the effect of drug treatment on spontaneous motor activity and cutoff of the photocell beam was recorded.

**Motor coordination activity**

The motor coordination and performance of each rat was evaluated using rota-rod apparatus. Latency to fall from the rotating bar was registered in seconds.

**Hot plate test**

In this test, animals were individually placed on a hot plate with the temperature adjusted to 43°C ± 1°C. The latency to the first sign of paw licking or jump response to avoid the heat was taken as an index of the pain threshold.

**Tail immersion (hot water) test**

The tail of the rat was immersed in a hot water bath (55°C ± 0.5°C) until withdrawal or signs of struggle were observed (cutoff 10 s). Shortening of the tail-withdrawal time indicated hyperalgesia.

**Analysis of blood glucose and glycosylated hemoglobin level**

The blood glucose level was analyzed with glucometer using glucose reagent strip while HbA1c level was measured as reference cited.

**Results:**

**Table 1:** Effect of Supercritical carbon-dioxide fluid extract and normal ethanolic extract administration on Photoactometer test Number of cut off observed (seconds), Rota rod fall latency from bar, hot plate jump time, Tail immersion test

| | **Photoactometer test Number of cut off observed (seconds)** | **Rota rod fall latency from bar** | **Hot plate jump time** | **Tail immersion test Tail withdrawal latency before treatment (s)** | **Tail immersion test Tail withdrawal latency before treatment (s)** |
|---|---|---|---|---|---|
| **Normal control** | 109.17± 6.94 | 36.67±2.21 | 4.33±0.33 | 6.5±0.22 | 5.33±0.33 |
| **Negative control** | 63.83±3.80 | 5.67±1.49 | 2.5±0.22 | 3.5±0.34 | 2.67±0.33 |
| **Test Group (250mg/kg)** | 88±1.06 * | 17±0.86* | 7±0.26* | 4.17±0.54 | 6.50±0.43* |
| **Test Group (500mg/kg)** | 89.66±2.25* | 16±0.82* | 6.83±0.31* | 5.67±0.33 | 7.33±0.33* |
| **Standard group** | 83.67±9.55* | 12.83±0,6* | 6±0.26* | 4.83±0.17 | 6.33±0.42* |
| **Test Group (250mg/kg)** | 75.00±2.05* | **9.33±0.95** | **3±0.26** | 5±0.26 | 4.83±0.60* |
| **Test Group (500mg/kg)** | 76.66±0.88** | 11.83±1.9* | 3.83±0.17* | 5.83±0.17 | 4.33±0.21** |

*The result is significant at $p < .01$ when test and standard group compared with Negative control group One-Way ANOVA Calculator, Including Tukey HSD

**The result is significant at $p < .05$ when test group compared with Negative control group One-Way ANOVA Calculator, Including Tukey HSD

Diabetic animals showed reduced locomotion ability as observed in a number of cut off significantly different from normal control rats. Supercritical carbon-dioxide fluid extract treated diabetic animals showed a significant rise in locomotor time as compared to negative control rats. Normal ethanolic extract treated diabetic animals showed a significant rise in locomotor time as compared to negative control rats. Supercritical carbon-dioxide fluid extract was found to more superior than normal ethanolic extract.

The rota-rod test experiment demonstrated the impairment of the motor function and coordination in the diabetic rats with a significant reduction in fall off time as compared to normal control rats. Supercritical carbon-dioxide fluid extract treated diabetic rats showed a significant increase in fall off time as compared to diabetic animals. Normal ethanolic extract treated diabetic rats showed a increase in fall off time as compared to diabetic animals. Results of supercritical carbon-dioxide fluid extract was found to more statistically more significant than normal ethanolic extract.

STZ injected rats had nociceptive threshold significantly lower than normal control rats as observed by tail immersion test and hot plate assay by decrease in tail withdrawal latency and jump time on hot plate. Supercritical fluid extract and normal ethanolic extract of seeds of *Vigna mungo* treated diabetic rats exhibited a rise in the tail withdrawal latencyas compared to diabetic control. Extract treated group showed significantly improved pain threshold, while Results of supercritical carbon-dioxide fluid extract was found to more statistically more significant than normal ethanolic extract.

**Table 2** Effect of Supercritical carbon-dioxide fluid extract administration on Mean Glycosylated hemoglobin level and glucose level

| | Glycosylated hemoglobin % | Glucose level Mg/dl |
|---|---|---|
| **Normal control** | 3.71±0.56 | 123.17±12.43 |
| **Negative control** | 6.57±1.29 | 403.17±30.32 |
| **Test Group (250mg/kg)** | 4.02±0.30* | 273.83±60.23* |
| **Test Group (500mg/kg)** | 3.97±0.54* | 214.00±73.33* |
| **Standard group** | 4.42±0.79# | 152.83±29.17# |
| **Test Group (250mg/kg)** | 4.75±1.25* | 291.33±96.31** |
| **Test Group (500mg/kg)** | 5.32±0.68* | 258.33±30.59* |

- *The result is significant at $p < .01$ when test and standard group compared with Negative control group One-Way ANOVA Calculator, Including Tukey HSD
- **The result is significant at $p < .05$ when test and standard group compared with Negative control group One-Way ANOVA Calculator, Including Tukey HSD

**Effect of supercritical fluid extract and normal ethanolic extract of seeds of *Vigna mungo* on blood glucose level:** sharp rise in negative control group than normal group blood glucose level is seen at end of the study. At the end study statistically significant fall is seen in blood glucose level of test groups. Supercritical fluid extract of seeds of *Vigna mungo* on mean Blood glucose level dose dependent action is seen. Further more significant fall in blood glucose level is seen in standard group than test group at the end of study. Results of supercritical carbon-dioxide fluid extract was found to more statistically more significant than normal ethanolic extract.

**Effect of Supercritical fluid extract and normal ethanolic extract of seeds of *Vigna mungo* on Glycosylated heamoglobin**: significant rise in glycosylated heamoglobin level in negative control group than normal group is seen at the end of forty five days. Supercritical fluid extract of seeds of *Vigna mungo* significantly reduced Glycosylated hemoglobin, level in both test groups. Dose dependent action is seen. Further more significant reduction in standard group is seen of glycosylated heamoglobin. level.

**Discussion**

In the present study *Vigna mungo* Supercritical carbon-dioxide fluid extract prepared. Supercritical carbon-dioxide fluid method is used due to the non-toxic nature of fluids used such as $CO_2$.Supercritical fluid extraction using $CO_2$ yielded distinct extracts of superior quality[13].The use of $CO_2$ will allow for collecting higher amounts of phenolic compound from plant seeds. It gives advantages over conventional extraction method like soxlet and maceration. Carbohydrate, Protein, Alkaloids, Flavonoids, Terpenoids, Steroids, Phenols and Glycosides were found in this extract indicates its possess medicinal value. The above said extract tested for antidiabetic activity which was not previously reported. Diabetes induction was done by standard method using streptozotocin. Streptozotocin damages pancreatic beta cells and produces hyperglucemia, hyperinsulinemia. This causes reduction in beta cell number permanently due to alkylating property of streptozotocin. Sterptozotocin inhibit glucose induced insulin secretion[14, 15, 16]. Supercritical carbon-dioxide fluid extract reverses hyperglycemic condition effectively in diabetic rats. Glibencamide is standard antidiabetic drug leads to increase in insulin secretion. Results of supercritical carbon-dioxide fluid extract was found to more statistically more significant than normal ethanolic extract.

Glycosylated hemoglobin indicates high blood sugar level, which may be fatal for eye and feet[17.]Supercritical carbon-dioxide fluid extract decreases its level and improvement is seen in it due to its medicinal value.

Increase in glycosylation of proteins including hemoglobin is seen with uncontrolled or poorly controlled diabetes. The glycation of myelin protein may contribute to the impairment of nerve conduction. These advanced glycation end products are also present in peripheral nerves which could interfere with axonal transports[18]. There is loss of pain perception in diabetes probably due to nerve damage and induction of peripheral neuropathy [19, 20]. Thermal hypoalgesia has been reported in diabetic rats using the tail-flick test or the hot-plate test[21, 22]. In this study, streptozotocin-induced diabetic control rats showed significant hypoalgesia in the hot-plate and tail immersion method. The delay in tail withdrawal response depicts the involvement of spinal reflex arc. Hypoalgesia was more significant in curative than preventive group, indicating long-term diabetes causes more hypoalgesia. Previous studies also have shown similar hypoalgesia [23]. Treatment with extracts and standard drug showed a significant decrease in the tail withdrawal response and increase in pain sensitivity when compared with diabetic control

group, suggesting protective effect against neuropathy. The probable mechanism could be via enhancing insulin production and decreasing the glucagon production. The onset of neuropathic complications could be prevented by early glycemic controls. At the same time a progress in locomotor activity and significant increase in fall off time was also observed. This indicates its protective role against damage to the neurons. Devlopment of diabetic neuropathy developed which was consistent with previous reports [24, 25]Results of supercritical carbon-dioxide fluid extract was found to more statistically more significant than normal ethanolic extract indicates supercritical extract possess greater medicinal value[26-43].

## Conclusion

Studies revealed that Supercritical fluid extract of seeds of *Vigna mungo* can be considered as an important addition to the therapeutic approach for the treatment of diabetic neuropathy. Further studies can be undertaken at the cellular and molecular level, which may further elucidate its mechanism in detail.

## Disclosure

The authors declare that there is no conflict of interest.

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