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Isolation of Bioactive Constituents from *Trigona iridipennis* Including Its Metabolic Products for *In Vivo* Pharmacological Screening for Anti-Parkinsonism

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Biju C.R., Ayswarya K., Jyothisree G*., Arunlal V.B., Babu G.

Department of Pharmaceutical Chemistry, Devaki Amma Memorial College of Pharmacy, Chelembra, Malappuram, Kerala- 673643. India.

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ABSTRACT

Trigona iridipennis is a rich source of phyto constituents, which are used against various disorders and possess various pharmacological activities. Parkinson's disease is a progressive neuro degenerative disorder that occurs due to dopamine deficiency in brain. The purpose of the present study was to identify the bioactive constituents from *Trigona iridipennis* extract, including its metabolic products for anti-Parkinsonism activity. Continuous soxhlet extraction was carried out by using 70% ethanol and isolation was carried out by column chromatographic method to find out the various bioactive constituents present in the extract. The isolated compound was structurally characterized by IR, NMR and Mass spectroscopic methods. The acute oral toxicity results showed that the isolate was found to be safe up to 2000 mg/kg. The isolated compound at a dose level of 200mg/kg, 400mg/kg were selected for evaluating antiparkinsonism activity by Block test, Rotarod test and Tardive dyskinesia test. Scopolamine 2mg/kg and combination of L-Dopa and Carbidopa(10mg/kg) were used as standards. It showed a significant antiparkinsonism activity at different dose levels. Proanthocyanine is the major compound which is responsible for anti-Parkinsonism activity.

INTRODUCTION

Trigona iridipennis is the most common dammer bee in the country. Besides *Trigona iridipennis*, that is common all over India, three other species occur in the Khasi hills of Meghalaya: *T. iridipennis, T. ruficornis and T. arcifera*[1]. In all over the world different types of honeybees are present but only seven species and 44 subspecies are recognized. *Trigona iridipennis* is a type of bees which is commonly seen in tropical areas of Kerala, it produce honey which have some medicinal values like common bees. This species can be identified by its middle thorax region with four distinct hair bands separated by broad glabrous interspaces[2]. It can be further distinguished from other species in its sub group by its chestnut brown coloured mandible which does not have a black apical area and a few dark brown erect setae or hair-like structures on the margin of scutellum. Based on the studies of workers *T. iridipennis* has a total body length ranging from 3.5 – 4 mm and entire body which is black to blackish brown. Size differences as well as male genitilia are also key for identifying the species[3, 4].

Products of Stingless bees are highly medicinal because they collect nectar and pollen selectively from medicinally important small herbal plants and flowers such as Coco palm, banana, guava, papaya, mango tamarind, thumba-poo, thengen-boo, touch-me- not plant, jack fruit tree, tulsi, teak etc., They are the most important pollinators of treasured herbal plants. Since stingless bees do not have sting and lacks defense organs, it protects the medicinally important rich food resources by covering the larger holes of the hive with wax-like substance and seals the minute pores of the hive using a special type of resinous substance which it creates on its own by mixing its own body secretion from the salivary glands with the resins collected from the leaves, trees, plants, buds etc. This natural resinous substance produced by bee secretion and substances collected from plant parts is called propolis[5]. Propolis have a wide spectrum of pharmacological activities such as antibacterial, antimicrobial, antioxidant, anti-herpes, antiulcer, antihypertensive, anti- inflammatory and also possess anticancer properties[6,7,8].

Parkinson's disease is a progressive neurodegenerative disease that occurs due to dopamine deficiency in brain. Parkinsonism is the second most common neuro - degenerative disorder affecting at least 2% of the world wide population aged 65 and older. it is characterized by a selective loss of dopaminergic neurons in the substantia nigra pars compacta region of the midbrain that culminates in the major clinical symptoms of parkinson's disease. The

symptoms of Parkinson's disease appear when there is a loss of about 50% dopaminergic neurons in the brain. The causes for dopaminergic neurons damages are not well understood, emerging data from recent studies shows that the adversed interaction between the neurotoxins formed from the environment, mitochondrial complex-1 inhibition, and different types of cells damages like excitotoxicity, inflammation, apoptosis, inflammation, protein aggregation and distressed energy metabolism[11, 12].

This work is aimed to isolate the bioactive constituents responsible for anti Parkinsonism activity from Trigona iridipennis and its metabolic products. The anti Parkinsonism activity was evaluated by *invivo* methods such as block test, rotarod test and tardive dyskinesia test.

MATERIALS AND METHODS

Collection and authentication of *Trigona iridipennis*

The fresh species of *Trigona iridipennis* was collected in the month of October from Nilambur area and authenticated.

Extraction

The fresh bees of *Trigona iridipennis* was killed and dried in the shade. A sample of hydro alcoholic bees extract was prepared by continuous hot percolation method for 3 hrs, by using a Soxhlet apparatus. About 500 grams of bee's material packed uniformly into a thimble and extracted with 1000 ml of hydro alcohol (70% ethanol + 30% water). The extracts were filtered and concentrated to dry mass at room temperature.

Phytochemical identification tests

The hydro alcoholic extract was subjected to qualitative chemical tests for the detection of various plant constituents like carbohydrates, glycosides, proteins and amino acids, fixed oils and fats, gums and mucilage, alkaloids, phytosterols, flavanoids, tannins and phenolic compounds, saponins, triterpenoids, etc.

Column chromatography

The hydroalcoholic bees extract of *Trigona iridipennis* was subjected to column chromatography for the separation of phytoconstituents. A column of suitable size (1 m × 1.5 inch) was chosen and packed with silica gel 100-200 mesh by adding slurry of the adsorbent

in petroleum ether. The extract of *Trigona iridipennis* was dissolved in ethanol and mixed with silica gel and fed to the column through a funnel. Petroleum ether was added to the column and kept aside without disturbance for the settlement of the extract. Maximum precautions were taken to remove the air bubbles. The column was eluted with different organic solvents in the order of increasing polarity. Fractions showing similar R_f value and identification test, were pooled together and solvents evaporated to get residues.

Spectral characterization

The isolated compound was characterized by IR, NMR and Mass spectroscopy and determined the chemical structure of the compound.

Pharmacological studies

Swiss albino male mice weighing 25- 30 g were used for the study. The animal's experimental protocol has been approved by our Institutional Animal Ethics Committee (IAEC) with registration no: DAMCOP/IAEC/020.

Acute oral toxicity

The acute oral toxicity study was carried out on Swiss Albino mice as per guidelines No: 425 given by the organization for Economic Co-operations and Development (OECD 425, 1988). A limit test at one dose level of 2000 mg/kg. body weight was carried out with five animals and they have fasted overnight. Animals were observed individually after dosing at least once in the first 30 minutes periodically during the first 24 hrs, with special attention given during first 4 hrs and daily thereafter for 14 days. After the experimental period, the animals were weighed and humanely killed and their vital organs including heart, lungs, liver, kidneys, spleen, adrenals, sex organs, and brain were grossly examined. The standard procedures were carried out as per OECD, 425 guidelines.

***In vivo* Anti Parkinsonism Study**

Block test

Male Swiss Albino mice weighing 25-30 g were divided into five groups of six animals each (n=6).

Group 1 - 0.5% w/v CMC 1ml/100gm orally for 10 days

Group 2 - 0.5% w/v CMC 1ml/100gm orally for 10 days +

Haloperidol 2mg/kg i. p. in water for injection

Group 3 - Scopolamine (1mg/kg) orally for 10days + Haloperidol i.p

Group 4 - Isolate of *T. I.* (200mg/kg) orally + Haloperidol i. p.

Group 5 - Isolate of *T. I.* (400mg/kg) orally + Haloperidol i. p.

The isolate was administered orally for ten days. On tenth day, after one hour, haloperidol was given I.P to induce catalepsy. After half an hour of the administration of Haloperidol, the animals were taken to measure the catalepsy. Same pattern was followed for Scopolamine which was administered I.P. Once the experiments were over, the animals were rehabilitated.

Test for rigidity (Rotarod test)

Male Swiss Albino mice weighing 25-30 g were divided into five groups of six animals each (n=6).

Group 1 - 0.5% w/v CMC 1ml/100gm orally for 7 days.

Group 2 - 0.5% w/v CMC 1ml/100gm orally + Haloperidol i. p.

Group 3 - L-Dopa + Carbidopa (10mg/kg) i. p. for 10days + Haloperidol i. p. in water for injection .

Group 4 - Isolate of *T. I* (200mg/kg) orally + Haloperidol i. p.

Group 5 - Isolate of *T. I.* (400mg/kg) orally + Haloperidol i. p.

The main symptom of Parkinsonism disease is muscle rigidity. This effect can be easily studied in animal by using rotarod apparatus. Before the test, each animal was given 1 min exposure to the moving rod. The animal was placed on the rotating rod for 3 min. Latency to fall off from the rotating rod of animal in control and the treated group was recorded. Movement impairement was indicated by the inability of the animal to remain on the rotating rod for a 3 min test period. Once the experiments were over, the animals were rehabilitated.

Test for tardive dyskinesia

The animals were divided into five groups, each group have six animals same as above tests.

Tardive dyskinesia is referred to as Vacuous Chewing Movements (VCMs) in rodents. On the test day mice were placed individually in a small cage for the assessment of oral dyskinesia. Animals were allowed 10min to get used to the observation cage before behavioural assessments. In the present study VCMs are referred to as single mouth openings in the vertical plane not directed toward physical material. The behavioral parameters of oral dyskinesia were measured continuously for 5 min.

Statistical Analysis

Results of the above experiments were expressed as Mean± SEM, and the difference between mean was analyzed by analysis of variance (ANOVA) using graph pad prism followed by Dunnet test, with $P<0.05$ being considered as statistical significant.

RESULTS AND DISCUSSION

Extraction

The continuous soxhlet extraction was carried using hydro alcohol as solvent and the percentage yield was found to be 45% w/w. The phytochemical studies of hydro alcoholic extract of *Trigona iridipennis* showed the presence of carbohydrates, flavonoids, proteins and amino acids, vitamin C, sterols and triterpenoids and saponins.

Column chromatography

The hydroalcoholic extract was subjected to column chromatography using different solvents and in that major fractions were obtained in n- butanol: ethanol (90:10) mobile phase. The fractions were confirmed by thin layer chromatography. The isolated compound were identified by using n-butanol: acetic acid: water (4:1:5) combination mobile phase and iodine chamber as detecting agent. Rf value obtained was 0.742, which was similar to the Rf value of flavanoids and it was confirmed.

Spectral Characterization

Infrared spectroscopy

The structure of *Trigona iridipennis* was elucidated by using Fourier Transform Infra-Red (FTIR) spectrophotometer (Jasco - 4600). The IR values are measured in cm^{-1}. The peak at a region $3658cm^{-1}$ indicates the presence of hydroxyl group in the isolated compound. The peak at a region $3100cm^{-1}$ indicates the presence of aromatic moiety in the isolated compound. The peak at a region $3010cm^{-1}$ indicates the presence of –CH stretching. The peak at a region $1400cm^{-1}$ indicates the presence of C=C stretching. The peak at a region $1231cm^{-1}$ indicates the presence of COC stretching.

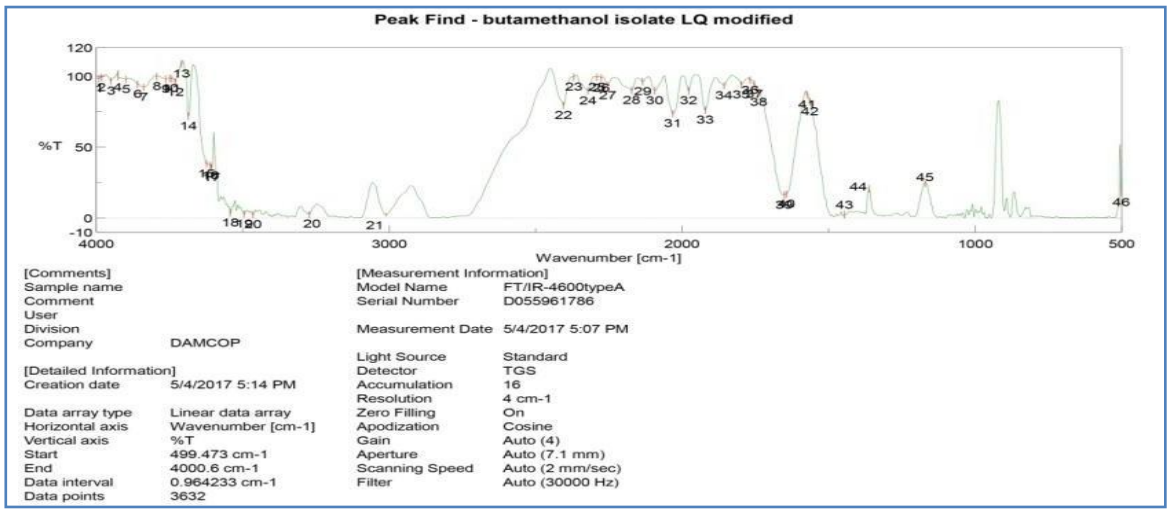


Figure 1: IR spectra of an isolated compound

NMR spectroscopy

The sample is dissolved in chloroform and value is measured in δ ppm.

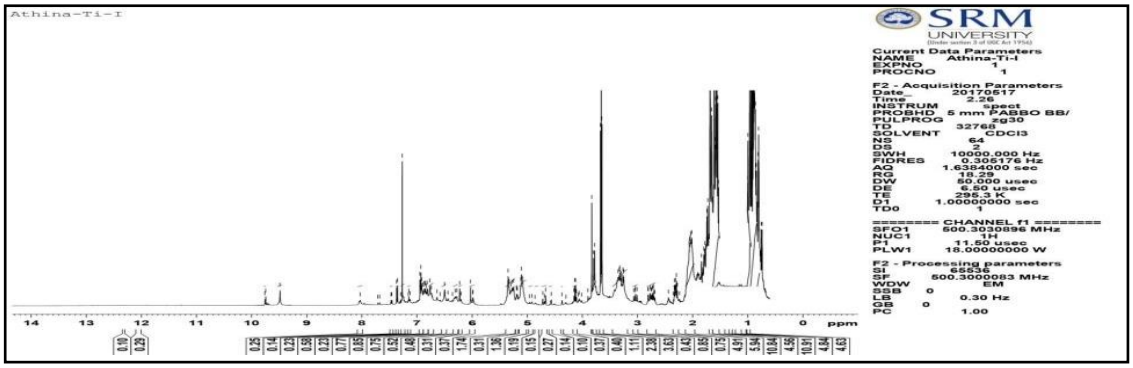


Figure 2:^{1}H NMR spectra of isolated compound

The singlet at 5.0 δ ppm indicates the presence of protons as –OH group attached to benzene ring. The multiplet at 2.83 δ ppm indicates the presence of protons as CH_2 group. The singlet at 5.75, 5.66, 5.71, 6.49 δ ppm indicates the presence of protons attached to benzene ring. The singlet at 5.05 δ ppm indicates the presence of protons as methane group. The singlet at 5.66 δ ppm indicates the presence of protons as benzene ring. The singlet at 6.49 δ ppm indicates the presence of protons as pyran ring.

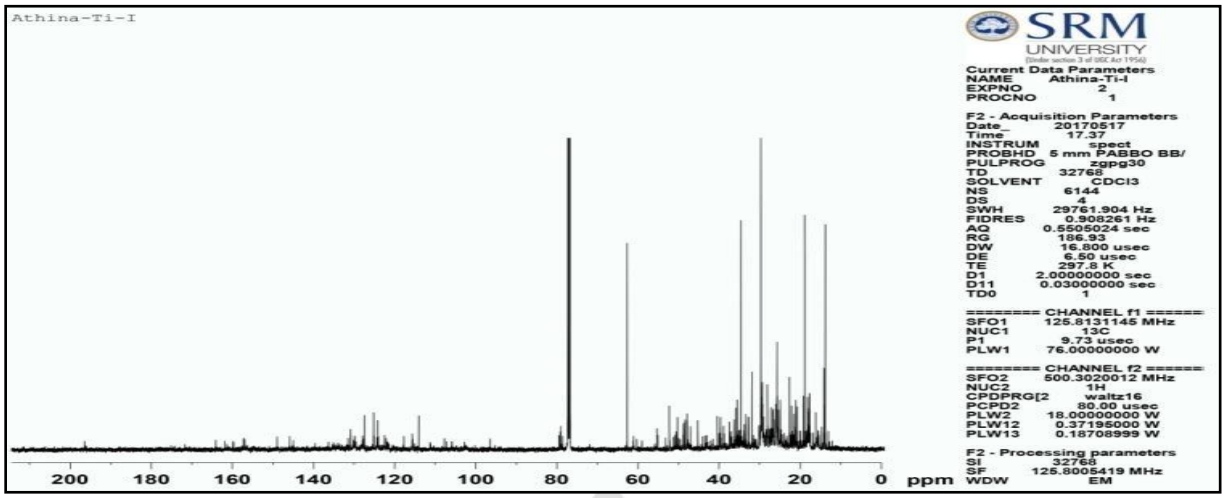


Figure 3:^{13}C NMR spectra of isolated compound

The signal at 29.2ppm indicates the presence of 1^0 carbon. Signal at 79.5ppm indicates the presence of 2^0 carbon; this may be attached to oxygen atom. Signal at 157.5ppm indicates the presence of 3^0 carbon may be attached to oxygen atom. The signal at 158.0, 105.1, 154.9, and 144.6ppm indicates the presence of 2^0 carbon attached to –OH group. Signal at 79.8ppm indicates the presence of 3^0 carbon attached to pyran ring.

Mass spectroscopy

The structure of *Trigona iridipennis* isolate was elucidated by using LC-MS (LC- MSD TRAP-SL 2010 A-SHIMADZU).

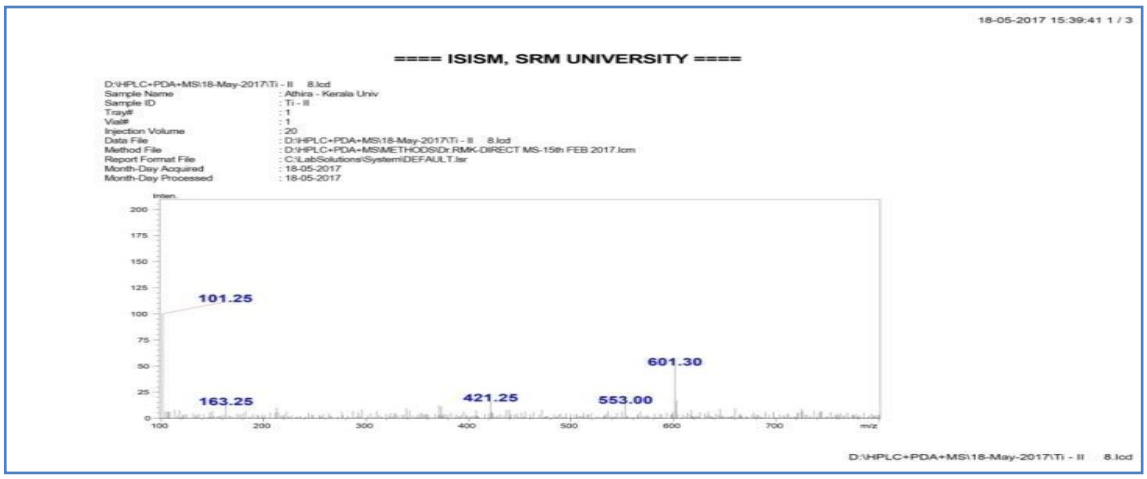
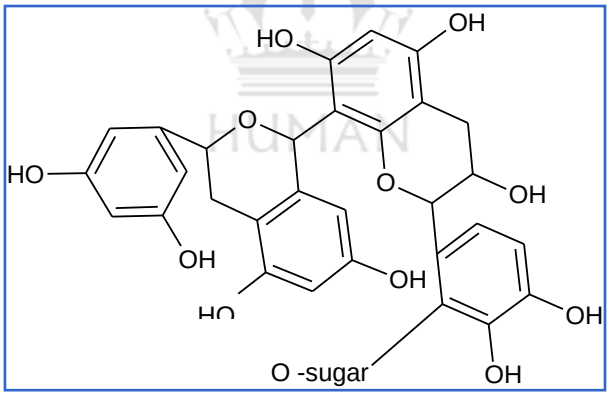


Figure 4: Mass spectrum of isolated compound

The base peak was shown at 101.25m/z and molecular mass is 601.30.

As per the various observations of chemical test and interpretation of IR, ^{1}H NMR, ^{13}C NMR and mass spectra, the expected structure of isolated compound was found to be Proanthocyanidin.



Acute oral toxicity study

Isolate was dissolved in CMC and administered orally and observed for 14 days. No mortality or no gross behavioral and weight changes for 2000mg/kg of *Trigona iridipennis* isolate, which showed that the isolated compound was safe up to a dose of 2000mg/kg.

***In vivo* methods**

Block test

In block test method, the time taken for withdrawal of both front paws from the block was

measured. The group which received Haloperidol, significantly increases the time taken for withdrawing paws which was seen on 10th day as compared to the normal group. In the standard treated group, a significant decrease in time taken for withdrawing the paws was seen as compared to the Haloperidol treated group. In an isolated treated group, a significant decrease in time taking for withdrawing the paws was seen on 10th day of treatment as compared to the Haloperidol treated group. Whereas no significant difference in time taken for withdrawing the paws was seen when 400mg/kg treated group compared to standard treated group.

Table 1: Effect of isolate during antiparkinsonism study by block test

Treatment	**Withdrawal of both front paws in minutes**
Control	0.38±0.0021
Haloperidol	35.16±0.3073
Scopolamine + Haloperidol	1.15±0.0223***
Test (200mg/kg)	5.56±0.0333**
Test (400mg/kg)	2.53±0.0210**

All values are expressed as mean ± S.E.M, n=6 in each group. ***P<0.001; **P < 0.01.

Rota rod test

In Rotarod test, the group which received only Haloperidol, significantly decreased falloff time which was seen on 1st, 4th and 7th day as compared to the normal group. In standard treated group, a significant increase in falloff time was seen on 1st, 4th and 7th day as compared to the Haloperidol treated group. In isolate treated group, a significant increase in falloff time was seen on 1st, 4th and 7th day as compared to the Haloperidol treated group. Whereas no significant difference in falloff time was seen in when 400mg/kg treated group compared to standard treated group.

Table 2: Effect of isolate on rotarod test

Treatment	Fall of time(sec)		
	Day 1	Day 4	Day 7
Control	70.5±0.4282	70.83±0.3073	71.16±0.4773
Haloperidol	22±0.3651	26.5±0.4282	23.16±0.3073
L-Dopa + Carbidopa	52.66±0.4944***	51.33±0.2108***	52.16±0.4773***
Test (200mg/kg)	32±0.3651**	29.33±0.4944**	31.66±0.4216**
Test (400mg/kg)	45.5±0.4282**	40.66±0.4216**	43.66±0.3333**

All values are expressed as mean ± S.E.M, n=6 in each group. **P<0.01, ***P<0.001.

Tardive dyskinesia test

In tardive dyskinesia test, the group which received only Haloperidol, significantly increase the number of vacous chewing movements on 10th day of the treatment as compared to normal group. In standard treated group, a significant decrease in number of vacous chewing movements as compared to Haloperidol treated group. In isolate treated group, a significant decrease in number of vacous chewing movements as compared to Haloperidol treated group. Whereas, no significant difference in falloff time was seen when 400mg/kg treated group compared to standard treated group.

Table 3: Effect of isolate on tardive dyskinesia test

Treatment	**VCMs in 5min on 10thday (mean ± SEM)**
Control	5.5±0.2236
Haloperidol	40.5±0.2236
Scopolamine + Haloperidol	12.16±0.2236***
Test (200mg/kg)	28.5±0.2236**
Test (400mg/kg)	20.5±0.2236**

All values are expressed as mean ± S.E.M, n=6 in each group. ***P<0.001, **P<0.01.

SUMMARY AND CONCLUSION

The present study mainly focused to isolate the bioactive constituents from *Trigona iridipennis* for antiparkinsonism activity. The bees are authenticated and subjected to

extraction by continuous hot soxhlet extraction method using hydro alcohol as a solvent and the extract was subjected to phytochemical analysis and it showed the presence of carbohydrates, flavanoids, saponins, sterols and triterpenoids, vitamin C, proteins and aminoacids. The hydro alcoholic extract was subjected to column chromatography for the isolation of bioactive constituents in the ratio of n-butanol: ethanol (90:10) fraction. TLC of isolated compound was developed using n-butanol: acetic acid: water (4:1:5) and its R_f value were found to be 0.742. The isolated compound showed the presence of phytoconstituents such as flavanoid and carbohydrates.

The purified compound was structurally elucidated using IR, ^{1}H NMR, ^{13}C NMR and Mass spectral studies. The acute oral toxicity study of isolated compound was carried out as per the OECD guidelines 425 and it revealed that the compound is safe up to the dose level of 2000mg/kg body weight of animals and no mortality was observed among the animals used. The *in vivo* antiparkinsonism activity was performed for the isolated compound, against haloperidol induced Swiss albino mice. Haloperidol was induced at a dose of 2mg/kg body weight intraperitoneal injection. The study of *in vivo* antiparkinsonism activity was carried out by block test, rotarod test and tardive dyskinesia test. The results showed that the isolated compound shows better antiparkinsonism activity with two different doses. The isolated compound contains proanthocyanidin (flavanoids) as primary metabolite and sugars as secondary metabolite and the structure was found to be polyhydroxy flavan-3-ol with molecular formula $C_{31}H_{28}O_{12}$. The most of the flavonoids produce antiparkinsonism activity by protecting the dopaminergic neurons and activate endogenous antioxidants enzymes hence reducing the symptoms of Parkinsonism. Since the isolated compound is a flavanoid, it can activate dopamine receptor and produce antiparkinsonism activity.

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