In-Vivo Antioxidant Potentials Of Citrullus Colocynthis Schard In Rotenone Induced Parkinson's Disease Animal Model.

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Abstract: the current study aims at investigating the Neuroprotective efficacy of an herb INDRAVARUNI – Citrullus colocynthis Schard by estimating its effect on endogenous antioxidant molecules in rat brain samples, using Rotenone induced Parkinson's disease animal model. 42 healthy male Wistar rats were selected for the study and were divided into 7 groups. Before inducing Parkinson's disease, the experimental animals were acclimatized for three days. The Rotenone solution was administered at 2.5mg/kg body weight/day intraperitonealy to all the groups, except for the normal control group which received only the vehicle (DMSO + Triester F 810). The respective treatment groups received their treatment from the day of starting of rotenone injection. Animals developing debilitating phenotype – limiting their mobility, feeding or grooming; were sacrificed and brain samples procured from them. Ultimately the rats surviving till the 9th day were also sacrificed on 10th day and brain samples procured, which were used for estimating MDA, SOD, CATALASE and GSH levels. The BID and TDS dosage groups showed maximum efficacy in endogenous antioxidant parameters.

Keywords: Citrullus colocynthis, Antioxidant, Rotenone, Parkinson's disease.

INTRODUCTION

Citrullus colocynthis an herb belonging to Cucurbitaceae family is an annual or perennial desert viny plant that grows in sandy arid soils.[1] It is widely used in the Indian system of medicine for different ailments[2] and is specifically prescribed by a classical Ayurvedic text Sharangadhara Samhita in the management of KAMPA VATA in the form of oil base preparation called VARUNI TAILA.[3] The clinical picture of Kampavata has a close match with the features of Parkinson's disease.[4,5,6] Parkinson's disease is one of the most common forms of a group of progressive neurodegenerative disorders characterized by bradykinesia, rest tremor, muscular rigidity, shuffling gait, and flexed posture[6]. Parkinson's disease as such is characterized by; Loss of ~50–70% of the dopaminergic neurons in the substantia nigra pars compacta, a profound loss of dopamine in the striatum, & the presence of intracytoplasmic inclusions called Lewy bodies, which are composed mainly of α-synuclein and ubiquitin[7]. Oxidative stress appears to play an important role in the sporadic forms of PD. Endogenous sources of oxidative stress include; the free radicals produced by the metabolism of dopamine and melanin. Additional stress may come from defects in mitochondrial complex I of the oxidative phosphorylation chain[6]. Exposure of rats to the pesticide and complex I inhibitor rotenone reproduces features of Parkinson's disease, including selective nigrostriatal dopaminergic degeneration and α-synuclein-positive cytoplasmic inclusions[8]. The In-vitro antioxidant activity of Citrullus colocynthis in scavenging DPPH and ABTS radical is already screened by Zohra Marzouk, et,al. which showed varied efficiency of different plant parts depending on the particular assay methodology and on the extract[9]. Hence, the current study is carried out to determine the In-vivo antioxidant potentials of Citrullus colocynthis in the ROTENONE induced Parkinson's disease rat brains.

MATERIALS AND METHODS

Animals

Healthy, adult, male Wistar rats (150-250g) were obtained from the Central animal house facility from J.S.S College of Pharmacy, S.S.Nagar, Mysore. The animals were kept in a well ventilated room and the animals were exposed to 12 hrs day and night cycle with a temperature between 20±3^{0}C. The animals were housed in large spacious, hygienic polypropylene cages during the course of the experimental period. The animals were fed with water and rat feed ad libitum. All the experiments were performed after obtaining prior approval from CPCSEA. The animals were housed in suitable environmental conditions.

Chemicals

The chemicals, which were used for the present study, were procured from Sigma Aldrich USA, Indian commercial company PVT.Ltd, Mumbai, Merk chemicals Mumbai.

Collection of plant material and preparation of Varuni Taila

Roots of botanically identified Citrullus colocynythis were collected from its natural habitat. The roots were cleaned properly and were macerated well to prepare a paste. One part of this paste was added with 4 parts of sesame oil and 16 parts of water. This mixture was cooked on mild fire till the oil part remains, and then was filtered and stored in a glass container.

Study design

Sample - 42 male Albino Wistar rats of middle aged group were selected for the study and were separated randomly into 7 groups of 6 animals in each. (Table 1.1)

Sl. No	Groups	No. of Rats	Inducing PD	Treatment
1	Negative Control	6	Rotenone + 98%Triester F 810 + 2% DMSO	Not Treated
2	Normal Control	6	Only 98%Triester F 810 + 2% DMSO	Not Treated
3	Vehicle Control	6	Rotenone + 98%Triester F 810 + 2% DMSO	Taila (Plain sesame oil – 1.34ml/Kg)
4	Test group A	6	Rotenone + 98%Triester F 810 + 2% DMSO	Varunitaila (1.34ml/Kg) OD dose
5	Test group B	6	Rotenone + 98%Triester F 810 + 2% DMSO	Varunitaila (1.34ml/Kg) Bid dose
6	Test group C	6	Rotenone + 98%Triester F 810 + 2% DMSO	Varunitaila (1.34ml/Kg) Tds dose
7	Standard Treated	6	Rotenone + 98%Triester F 810 + 2% DMSO	Standard dose (10mg/Kg) of L-Dopa

Table 1.1: *Depicting various groups employed and their respective treatments.*

Preparation of Rotenone solution

Rotenone solution was prepared as a stock for 3 days in 100% Dimethylsulfoxide (DMSO) and diluted in medium chain triglyceride, Caprylic / Capric Triglyceride (Triester F 810) to obtain a final concentration of 2.5mg/ml rotenone in 98% Triester F 810 & 2% DMSO. Vortexing the solution creates a stable emulsion of the DMSO containing rotenone &Triester F 810. Fresh stock solution was prepared twice a week and stored in a vial protected from light. Vortexing of the vial several times before each injection was ensured to eliminate the possibility of settling[10].

Schedule of the procedure

Before inducing PD, the experimental animals were acclimatized for three days. The Rotenone solution thus prepared was administered at 1ml/kg body weight/day intraperitonealy to all the groups, except for the normal control group which received only the vehicle (DMSO + Triester F 810) [10]. The respective treatment groups received their treatment from the day of starting of rotenone injection[11]. During daily handling, animals were observed closely for the emergence of Parkinson's disease phenotype[10, 11]. Animals developing debilitating phenotype – limiting their mobility, feeding or grooming; were sacrificed and brain samples procured from them [7]. Ultimately the rats surviving till the 9th day were also sacrificed on 10th day and brain samples procured, which were used for the estimation of endogenous antioxidant enzymes and lipid peroxidation levels.

Preparation of post-mitochondrial supernatant

The animals were sacrificed by decapitation on the 10th day of treatment or when they develop debilitating phenotype. The brain was removed, dissected into 2 halves mid-sagitally and washed in cold normal saline and kept on ice and subsequently whattman filter paper, then weighed and homogenized in cold phosphate buffer (0.1 M, pH 7.4). The homogenates were centrifuged at 10000 rpm for 10 min at 4ºC and post-mitochondrial supernatant (PMS) was used for the estimation of Lipid peroxidation. The supernatant was again centrifuged at 15000 rpm for 1 hr at 4ºC and the supernatant obtained was used for further estimation of GSH, SOD and CAT.

Estimation of Superoxide Dismutase (SOD) [12] levels

The Superoxide Dismutase (SOD) levels was determined by the method explained by Misra.HP et al,.based upon the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline PH, Which was measured UV Spectroscopically. Inhibition of the chromogen formation by superoxide dismutase was linear with increase in enzyme concentration. 150µl of carbonate buffer was added to 50ml of tissue homogenate. 100µl of Adrenaline was added directly in cuvette after putting in UV cuvette holder. Absorbance was read at 480nm (A_0-A_{60}) (kinetic mode). The total protein content of the tissue homogenate was determined by standard curve of protein. The enzyme concentration has been expressed as U/mg protein, where 1U of the enzyme is defined as the amount of enzyme required to inhibit the rate of epinephrine auto-oxidation by 50% under the conditions of the assay.

Estimation of Catalase (CAT) Enzyme[13]

The Catalase enzyme in the brain samples was assayed using the methodology described by Aebi H et al., based on the ability of Catalase enzyme to decompose Hydrogen peroxide radical to water and oxygen molecule.

$$2H_2O_2 \xrightarrow{\text{CATALASE}} H_2O + O_2$$

In presence of CAT, H_2O_2 shows a continual decrease in absorbance when measured in UV range. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240nm. The difference in absorbance (ΔA 240) per unit time is a measure of the activity. 3ml of H_2O_2 + Phosphate Buffer solution was added to 50µl of tissue homogenate. The above solution was kept in cuvette and reading was taken at zero time and 120 minute (absorbance at 240nm). The extinction coefficient of 0.04 $mM^{-1}cm^{-1}$ was used to determine the specific activity of catalase. A unit of catalase is defined as the quantity, which decomposes 1.0 µM of H_2O_2 per min at pH 7.0 at 25^{0}C, while this H_2O_2 concentration falls from 10.3 to 9.2mM. The total protein content of the tissue homogenate was determined by standard curve of protein. The data was expressed as U/mg protein.

Estimation of Glutathione (GSH) [13] levels

The Glutathione (GSH) is a non-protein compound containing sulfyhydryl group in its structure. It is estimated in the brain samples using the methodology described by Aebi H et al., based on the principle that DTNB (Dithiobis2-nitro benzoic acid) is reduced by sulfyhydryl compound to an intensely yellow coloured compound. The absorbance of the reduced chromogen is measured at 412nm and is directly proportional to the GSH concentration. 500µl TCA solution was added to 500µl of tissue homogenate and then it was centrifuged. 500µl of supernatant was incubated with 3ml of Phosphate Buffer Solution and 500µl of DTNB for 10 mins at room temperature. Absorbance was read at 412nm (photometric method). The absorbance of the samples was recorded against the blank at 412 nm. GSH concentration was calculated from the standard curve by multiplying with the dilution factor.

Lipid peroxidation – Malondialdehyde (MDA) Levels[13]
Malondialdehyde formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. Malondialdehyde reacts with thiobarbituric acid to form red colour species (TBARS), which is measured at 535nm. 1ml of tissue homogenate was combined with 2ml of TCA-TBA-HCl reagent (15%w/v TCA, 0.375%w/v TBA and 0.2ml of 0.25 N HCl) and mixed thoroughly. The solution was heated for 15 mins in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 532nm against a blank that contains all the reagents minus the tissue homogenate. The malondialdehyde concentration of the sample can be calculated using extinction co-efficient of 1.56 x $105M^{-1}cm^{-1}$. Malondialdehyde concentration (M) = Absorbance at 532nm / 1.56 x 10.

Statistical Analysis
All the values were expressed as Mean ± SD. The data was statistically analysed using One-way ANOVA with Post Hoc Tukey's multiple comparison test using SPSS software. The values of $p < 0.01$ were considered as significant.

RESULTS

SOD Levels
Mean values of SOD levels expressed in U/mg protien is shown in Fig.1 Normal group showing a mean value of 108.0288333 U/mg protien is significantly different from rest of the groups. However Test group C with mean SOD levels 65.2195 U/mg protien has shown significant difference from Negative, Vehicle, Test Group A, Test group B and Standard control groups.

Catalase enzyme
Mean values of Catalase levels expressed in U/mg protien is shown in Fig.2 Normal group showing a mean value of 0.462833333 U/mg protien is significantly different from rest of the groups. And no group has shown statistically significant difference from the negative control group.

Glutathione Levels
Mean values of Glutathione levels expressed in µM/mg protien is shown in Fig.3 Normal group showing a mean value of 21.0258 µM/mg protien is significantly different from rest of the groups. And none of the groups show statistically significant difference from the negative control group at 99% confidence level. However if the confidence level is reduced to 95%, the Test group C Vs Negative control group has shown a significance level of 0.048 which is ≤ 0.05, hence the difference here could be considered as moderately significant.

Lipid peroxidation – MDA Levels
Mean values of MDA levels expressed in nM/mg protien is shown in Fig.4 Normal group showing a mean value of 13.3385 nM/mg protien is significantly different from rest of the groups. However the Test group B and C with mean MDA levels 90.24816667 nM/mg protien and 91.87333333 nM/mg protien respectively has shown significant difference from the Negative control group.

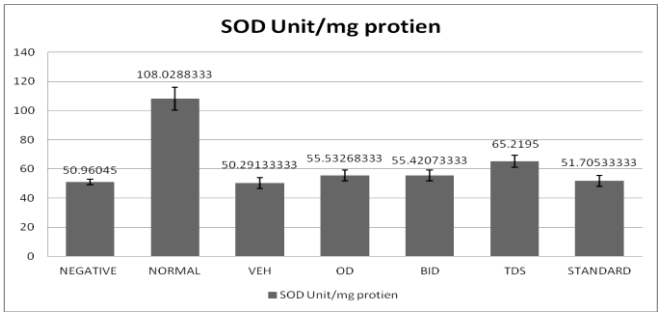

Figure 1: *Means of SOD levels represented in Unit / mg of protien.*

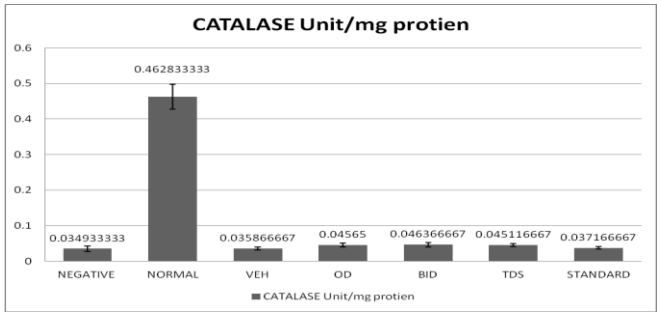

Figure 2: *Means of CATALASE enzyme levels in unit/mg protien.*

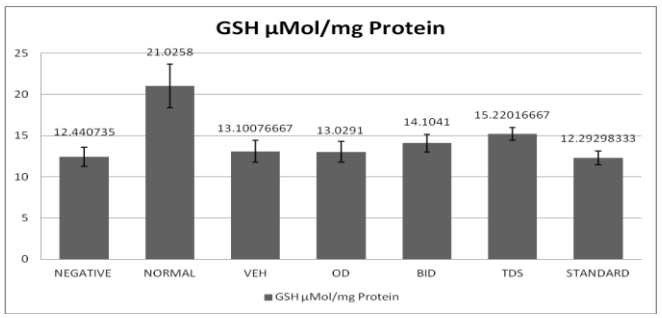

Figure 3: *Means of GSH levels in µM / mg of protien.*

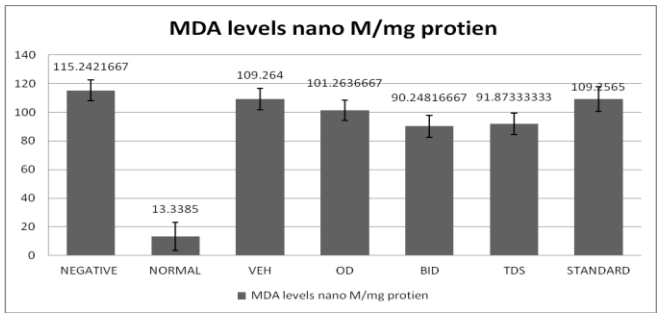

Figure 4: *Means of MDA levels in nM / mg of protien.*

DISCUSSION

Varuni Taila prescribed by an Ayurvedic text in the management of a clinical condition called Kampavata [3], whose features closely match with that of Parkinson's disease [4,5,6]. Parkinson's disease being a progressive neurodegenerative disorder, the current study is taken up to evaluate the neuroprotective efficacy of the test drug by estimating the endogenous antioxidant activity in rat brains. There are various animal models described which mimic Parkinsonian features, however the neurotoxin Rotenone is a

mitochondrial complex I inhibitor of the oxidative phosphorylation chain[6]. A similar pathway is also identified to be a cause for the production of oxidative stress in the pathogenesis of Parkinson's disease; hence Rotenone was selected for inducing Parkinson's disease in rats. The rat brain samples from all the groups were analysed for endogenous antioxidant enzymes and the results were subjected to statistical analysis. The SOD, Catalase and GSH levels were significantly high in the Normal control group when compared to other groups. This suggests that the neurotoxin Rotenone treatment has significantly lowered the endogenous antioxidant enzyme levels, as a result of mitochondrial dysfunction and increased oxidative stress. Meanwhile the Standard control group shows no difference (P value = 1.000) with the Negative control group with respect to SOD, Catalase and GSH levels, this suggests that the standard drug L-Dopa does not impart any effect on the neurotoxin induced oxidative stress. Even the Vehicle control (Taila administered) group and the Test group A (OD Dosage) also did not show significant difference with negative control group. With respect to SOD levels, the Test group C (TDS Dosage) shows significant difference from the groups Negative control, Vehicle control, Standard control, Test group A and Test group B. This signifies the efficacy of the test drug in TDS dosage to prevent the fall in SOD levels in rotenone treated rat brain samples. Catalase enzyme as concerned, none of the groups (other than normal control group) has shown any significant elevation. Hence it suggests that the test drug does not have any effect on Catalase enzyme elevation. The reason remain unclear, as described elsewhere by Zohra Marzouk, et,al. in an In-vitro antioxidant study which showed varied efficiency of different plant parts depending on the particular assay methodology and on the extract[9]. The varied phyto-constituents may impart varied effects on the endogenous anti oxidant enzymes depending upon their relative bio availability at the site of action. When coming to reduced Glutathione (GSH) levels, the Test group C (TDS dosage) has shown moderately significant difference from the Negative control group, suggesting some activity of the Test drug in its TDS dosage in elevating GSH levels. MDA levels are significantly elevated in all the groups when compared to the Normal control group. However the BID and TDS dosage of the Test drug has significantly inhibited the lipid peroxidation when compared to the Negative control group. The endogenous anti oxidant activity seen in the brain samples with respect to SOD and GSH levels can be attributed to the flavonoids present in the roots of Citrullus colocynthis. The recent researches have unleashed a number of potential mechanisms unrelated to the actual antioxidant capacity, by which flavonoids could account for at least part of the health benefits[14]. It appears that flavonoids are actually pro-oxidants under normal physiological conditions, i.e. they generate free radicals in small quantities[15,16]. This apparently harmful property however appears to up regulate the synthesis of antioxidant enzymes such as SOD, Catalase and Glutathione peroxidase, in what might be termed as "INDIRECT" antioxidant effect[17,18,19]. The concept of anti oxidant and pro oxidant effects is yet to be understood clearly. The homeostasis between these two is considered to be an important aspect of such an understanding.

CONCLUSION

The prescribed dosage of Varuni Taila in TDS frequency remained effective in elevating the SOD and GSH levels. While coming to lipid peroxidation the Bid dosage along with Tds dosage was also found effective in lowering the MDA levels. The OD dosage of Varuni Taila, plain Tila Taila and the Standard drug L-Dopa did not show any activity against the oxidative stress induced by the neurotoxin Rotenone in the current study.

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