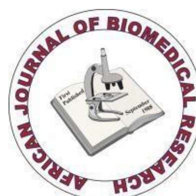

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Research Article

Effect of Stratified Doses of Murraya Koenigii on Brain Antioxidant Status of Wistar Rats Treated with Clozapine.

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Abstract
Clozapine is considered as the most potent antipsychotic medication for managing refractory schizophrenia. However, Clozapine may contribute to oxidative stress within the forebrain. Given that Murraya koenigii leaves are abundant in flavonoids and phenolic compounds, they possess significant free radical scavenging capabilities. Leaves were collected from southern parts of Goa, processed for extraction using Soxhlet extraction method with three solvents namely chloroform, methanol and water. The antioxidant property was evaluated in vitro using DPPH, ABTS, FRAP assays along with determination of MDA levels in all the three extracts. This study aims to examine possible outcome of Murraya koenigii on the Clozapine-induced oxidative stress in rats given saline, Clozapine, and Clozapine plus chloroformic extract of Murraya koenigii for 28 days. The brain was isolated and homogenized for the determination of Superoxide dismutase, Catalase, Glutathione peroxidase and lipid peroxidation assay. Our findings confirm the existence of hydrophilic polyphenolic compounds that contribute to the enhanced reducing capacity, along with elevated levels of flavonoids that are responsible for the ABTS and DPPH scavenging activities of the chloroform extract. In the clozapine-treated control group, oxidative stress was evident, characterized by a significant raise in MDA levels, simultaneously decreasing the levels of SOD, CAT and GPx when compared to the normal control. The CEMK-treated group exhibited an antioxidant effect, significantly reducing MDA levels and increasing antioxidant enzyme levels in comparison to the clozapine control subject in a dose dependent manner. So, this investigation makes it clear that the chloroform extract of Murraya koenigii leaves might significantly reduce the high levels of oxidative stress markers induced by the antipsychotic drug clozapine.

Keywords: Clozapine, Murraya koenigii, Antioxidant, Superoxide dismutase, Catalase, Glutathione Peroxidase, Lipid peroxidation, Flavanoids

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1. Introduction:

Antipsychotic medications, particularly Clozapine, serve as the principal pharmacological intervention for schizophrenia spectrum disorders and are being utilized more frequently for various other psychiatric conditions. However, Clozapine is linked to a wide range of adverse effects(1,2), which encompasses a diverse array of complications related to the immune system, metabolism, cardiovascular health, and mental health(3). Clozapine's hematologic issues, particularly the potential for agranulocytosis, have positioned it as the antipsychotic of "last resort"(4). Furthermore, there are side effects that often prompt physicians to implement interventions alongside ongoing treatment, such as tachycardia and seizures. As a result, different types of supplemental therapy, including physical activity, cognitive training, and procognitive or antidepressant drugs, have been proposed for the management of individuals with schizophrenia(5,6).

Lipids are dynamic regulators of numerous cellular processes like budding, signaling and a vital part of cell membrane. The abnormal lipid composition in membranes is regarded as a characteristic or susceptibility indicator linked to the disorder. Neuronal cell membranes make up the vesicular compartments that store and release neurotransmitter molecules, meaning changes in membrane lipid composition would be expected to impact neurotransmitter dynamics(7). Biomarkers of oxidative stress can reveal biochemical alterations within tissues, as various lipid categories have been recognized as significant intracellular signal transduction molecules(8). Unrestrained oxidative stress results directly into the damage of cells, tissues, and organs in their tissues by oxidative injury. Free radicals are well known for over twenty years as causes for causing direct lipid damage and being harmful when found at relatively high levels or ROS levels in an organism. Main places for the generation of intracellular ROS include the mitochondrion, plasma membrane, endoplasmic reticulum, and the peroxisomes(9).

Normally, antioxidant defenses by enzymes such as SOD, CAT and GPx protect against oxidative damage. SOD degrades superoxide radicals into hydrogen peroxide, which in turn is further degraded into water and oxygen by CAT and GPx to prevent the formation of hydroxyl radicals(10). Research involving clozapine has shown that it induces oxidative stress and causes oxidative damage to cells, which is demonstrated by increased levels of membrane lipid peroxidation and total protein oxidation observed in the brain and several other organs(11). Treatment with typical neuroleptics like haloperidol and fluphenazine was found to increase oxidative stress through alterations in antioxidant enzymes activity and related oxidative injury in the brain(10). Oxidative cell injury might be a contributory factor in the etiopathophysiology of schizophrenia itself, and this might be worsened by treatment with antipsychotics with pro-oxidant properties (12). Increased amount of TBARS was noted in Haloperidol treated rats but Clozapine was less associated with oxidative stress as demonstrated by Reinke et al (13). In addition to direct protein oxidation, antipsychotic treatment has been related with increased generation of reactive oxygen species (14).

Natural antioxidants are best derived from plants. Fruits and vegetables are a major source of bioactive metabolites and medicinal plants have been reported to play a key role in slowing down many of the pathogenesis and neurodegenerative disorders such as Alzheimer's and dementia(15) Numerous substances derived from plants, commonly referred to as "phytonutrients" or "phytochemicals," are gaining recognition for their positive impact on human health(16). In South East Asian countries, curry leaves are a widely utilized and popular spice herb(17) . In recent years, greater attention has been paid to the use of Murraya koenigii in traditional medicines and home remedies(18). Curry leaves have been a part of the human diet since ancient times, with no documented adverse effects (16). They are rich in vitamins, minerals, and other nutrients.(19) Our earlier studies reveal that aqueous, methanolic and chloroformic extracts of Murraya koenigii, contains phytochemicals Flavanoids, Tannins, Saponins, Glycosides, Phenols, and Terpenoids that exhibited antihyperlipidemic abilities through the inhibition of Pancreatic lipase(20). Leaf extracts of Murraya koenigii have also been reported to possess antifungal(21), antihypercholesteremic(22) and antioxidant activities(23). The presence of phenols and flavonoid derivatives in this plant suggests its potential as a source of potent antioxidant compounds, which may be beneficial in the treatment of human diseases caused by reactive oxygen species (ROS) (24). Among a few studies, Ethanolic extract of Murraya koenigii has been demonstrated to notably improve memory and protect against memory deficits induced by scopolamine (25). In another study, Murraya koenigii extract showed a modest enhancement in memory but did not significantly protect hippocampal neurons in rats suffering from chronic partial global cerebral ischemia.(26).

Considering that antipsychotic drug Clozapine presents oxidative stress during the course of its action, this study aimed to investigate the hypothesis that administering Murraya koenigii extract could reduce or reverse oxidative damage induced by a 28-day clozapine treatment, as measured by levels of lipid

peroxidation (TBARS) and the activities of catalase (CAT), superoxide dismutase (SOD) and Glutathione peroxidase(GPx) in the rat brain.

2. Materials and Methods

2.1 Sample collection and processing

Murraya koenigii leaf samples were collected from three different sites in South Goa. (Fig1) The leaves are washed with clean water thoroughly. This ensured no mud or foreign particles adhered to the leaves. Leaves were allowed to air dry for 2 weeks and then ground into a fine powder with the help of mechanical blender. All ground powder was kept in an airtight container in a dry and cool place till further use.

Fig 1 : Maps of South Goa showing collection site(27)

2.2 Extraction

The metabolites from the Murraya koenigii leaf sample was extracted using Soxhlet extraction method using solvents Chloroform, Methanol and water from non-polar to polar. In the Soxhlet extraction, the leaf material was continuously flushed with the fresh solvent. The principle of this method works by circulating fresh solvent through extractor containing powder (Coarse) by evaporation and subsequent condensation.

The crude extract was prepared using a Soxhlet apparatus with approximately 25 grams of dried and powdered leaves of Murraya koenigii, which had continuous operation through 10 reflux cycles. Three types of organic solvents-chloroform, methanol, and water-were chosen in order to have the free diffusion of solvents from these media into the plant material, which would dissolve its constituents. As a consequence, the process ensured that maximum bioactive compounds were extracted. Organic solvents were concentrated to dryness under reduced pressure with methanol evaporated at temperatures between 50ºC and 55ºC, chloroform at 40ºC to 45ºC and the aqueous extract at 80ºC to 87ºC, using a rotary evaporator. The extracts obtained were air dried and stored in refrigerator to use later(28).

2.3 Invitro Antioxidative activity

The antioxidant properties of Murraya koenigii were assessed through the evaluation of three different extracts, as plant antioxidants are utilized in the treatment of a range of diseases (chloroformic, methanolic and aqueous) with standard in vitro methods. These techniques rely on the suppression of free radical measurements, which can differ significantly depending on the type of radical produced, its consistency, and the final outcome.

2.3.1 Scavenging capacity of DPPH radical

In vitro, the free radical scavenging activity of the crude powder from Murrays koenigii was assessed using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) method. The effects of the samples on the DPPH radical were studied in detail according to the protocol of Von Gadow et al. (1997). A 2 mL aliquot of a 6×10^{-5} M methanolic DPPH solution was mixed with 50 μL of a methanolic sample solution containing 20 mg/mL. After mixing, absorbance readings were immediately taken, and the decrease at 515 nm was observed continuously with a spectrophotometer at room temperature for 16 min. Methanolic solutions of pure compound quercetin were also assayed with a concentration of 1 mg/mL. The scavenging effect is represented by the decrease in absorbance at 515 nm plotted against time. Percentage inhibition of DPPH

radicals for the sample was calculated at the absorbance values measured at the end of 16 minutes as given below(29). All measurements were conducted in triplicate, and the percentage inhibition of DPPH radicals by samples was determined by the formula devised by Yen and Duh (1994). The ability to scavenge the DPPH radical was expressed as percentage inhibition and calculated using the following equation: IP (%) = (A 0 - A 1) / A 0 × 100 where A0 is the absorbance of the control and A1 is the absorbance of the sample.

2.3.2 ABTS scavenging activity

The antioxidant in the extract bleaches the reaction mixture by decreasing the intensity of the colour and coverts ABTS Free radical to stable ABTS. This decolorization method of the ABTS radical cation was used by Re et al., to assess the scavenging activity of the extracts (30). Prepare a 7mM ABTS solution in water and oxidize it with 2.45mM potassium persulfate. Allow the mixture to settle in the dark for 12-16 hours, during which time it is ensured that the stability of the mixture has been established over a two-day period at room temperature. Prepare the ABTS*+ diluted with absolute ethanol to an absorbance of 0.702 at a wavelength of 730 nm. Equilibrate the solution at 30°C and then measure the absorbance. Mix 50µl of the plant extract at 20mg/ml with 2ml of diluted ABTS*+ solution and determine the absorbance at 734nm after exactly 6 minutes with the temperature at 30°C. Always include solvent blanks in the assay. The determination must be carried out at least three times using concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg/ml for reliability(30). The determination of ABTS is done by IP (%) = (A 0 - A 1) / A 0 × 100 where A0 is the absorbance of the control and A1 is the absorbance of the sample.

2.3.3 FRAP assay

The FRAP assay was conducted according to Benzie and Strain (1996) procedure with some modifications. A stock solution containing 300 mM of acetate buffer was prepared with 3.1 g of sodium acetate trihydrate and 16 mL of acetic acid while the pH was maintained at 3.6. It also included a 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) dissolved in 40 mM of HCl and a 20 mM $FeCl_3 \cdot 6H_2O$ solution. A fresh work solution was made through the combination of 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution, which was warmed to 37 °C before use. To the leaf extracts (150 mL), 2850 mL FRAP solution was added in a dark and kept aside for 30 minutes. The absorption of the colored product resulting was that of the ferrous tripyridyltriazine complex, measured at 593 nm. For accuracy in this experiment, measurements were carried out using concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg/mL for accurate estimation(31). IP (%) = (A 0 - A 1) / A 0 × 100 is used for the calculation of percent inhibition.

2.3.4 Lipid peroxidation

1 mL aliquot of the sample solution was mixed with 2 mL of 20% trichloroacetic acid and 2 mL of 0.67% 2-thiobarbituric acid. The mixture was then allowed to heat in boiling water bath for 10 mins. The mixture was cooled, and later centrifuged at 300 rpm for almost 20 mins. The absorbance of the resulting supernatant was measured spectrophotometrically at 550 nm(32).The concentrations of the samples tested are 0.2, 0.4, 0.6, 0.8, and 1 mg/ml. Inhibition percent is calculated by using the formula, IP (%) = (A 0 - A 1) / A 0 × 100 where A0 is the absorbance of the control and A1 is the absorbance of the sample.

2.4 In vivo antioxidative ability

The antioxidative ability of Murraya koenigii was assessed using animal model.

2.4.1 Procurement of animals

Wistar rats weighing 220-350 grams were taken for the study. Animals were procured from Lacsmi Biofarms, Pune. The rats were kept in husk covered propylene cages. Rats were retained in a 12 hours light/dark cycle at 25±2 ºC and 55±10 relative humidity. Rats were given regular feed and free access to water. The Institutional Animal Ethics Committee (IAEC), Goa University, Taleigao Plateau, Goa, India (Approval no: GUZ/IAEC/23-24/N1, Project code no. IAEC/23-24/N1) approved the study.

2.4.2 Study design

Clozapine (CLZ) was procured from Government pharmacy unit of Institute of Psychiatry and Human behavior, Bambolim, Goa. Clozapine was administered orally at the medication dosage of 10 mg/kg body weight for a span of 14 days to initiate oxidative stress. Chloroformic extract was chosen for in vivo studies due to the highest antioxidant capacity. Different doses of Chloroformic extracts of Murraya koenigii were administered orally 1-hour post clozapine treatment to the rats of the treatment groups. The extracts and the medication Clozapine were dissolved in distilled water and orally administered for 14 days. Dosages of the drug were calculated based on findings from previous research. The subjects were divided into six groups of six animals each. Group I was taken as the negative control and administered 10 ml/kg body weight of normal saline. Group-II served as positive control consisting of rats receiving Clozapine (10mg/kg bw). Group-III received Clozapine (10mg/kg bw) plus CEMK of 100mg/Kg bw after 1 hour of clozapine dosing. Group-VI received Clozapine (10mg/kg bw) plus CEMK (200mg/Kg bw after 1 hour). Group- V received Clozapine (10mg/kg bw) plus CEMK (400mg/Kg bw after 1 hour). Lastly the Group-VI received Clozapine (10mg/kg bw) plus CEMK (800mg/Kg bw after 1 hour).

2.4.3 Ex vivo assessment of antioxidant enzymes
Dissection and Homogenization:

Following the conclusion of the experiment, the rats were euthanized via cervical dislocation. The entire intact brain was meticulously extracted and placed on ice for cleaning, then stored at -20°C until further analysis. Rat brain tissue homogenate preparation was carried out where 1 gram of the wet tissue was

suspended with 10 milliliters of 0.05 M ice-cold phosphate buffer, pH 7.4, in weight-to-volume ratio and then homogenized with a Teflon homogenizer(33). Tissue homogenates underwent centrifugation at 15,000 ×g at a temperature of 4°C for a duration of 30 minutes. The resulting supernatant was subsequently filtered and preserved at −20°C until it was required for analysis (34). 0.2 mL of homogenate was utilized for estimation of TBARS. The residual portion of the homogenate was subjected to centrifugation at 15,000 rpm at a temperature of 4°C for a duration of 60 minutes, after which the supernatants were utilized for the estimation of superoxide dismutase (35).

Antioxidant profile of brain

a. Catalase (CAT) estimation

The CAT activities were determined by a reaction mixture comprising 2.5 ml of 50 mmol phosphate buffer at pH 5.0, 0.4 ml of 5.9 mmol H2O2, and 0.1 ml of tissue homogenate. The absorbance changes at 240 nm were recorded one minute after the preparation of the reaction mixture(34). A change in absorbance of 0.01 per minute per milligram of protein is regarded as one unit of catalase activity.

b. Glutathione peroxidase assay

The activity of glutathione peroxidase was assessed based on the protocol by Mohandas et al. in 1984. The assay mixture included 1.49 ml of phosphate buffer (0.1 mol; pH 7.4), 0.1 ml of EDTA (1 mmol), 0.1 ml of sodium azide (1 mmol), 0.05 ml of glutathione reductase (1 IU/ml), 0.05 ml of GSH (1 mmol), 0.1 ml of NADPH (0.2 mmol), 0.01 ml of H2O2 (0.25 mmol), and 0.1 ml of homogenate, making it 2 ml in volume. The decrease of NADPH was followed at 340 nm in an reaction temperature of 25°C. Enzyme activities were measured in nmol of NADPH oxidized per minute per milligram of protein, using a molar extinction coefficient of 6.22 × 103 $M^{-}1$ $cm^{-}1$ (34,36).

c. Superoxide Dismutase assay

SOD was estimated by inhibition of NADH - phenazine methosulphate nitroblue tetrazolium formazon complex. Method for estimating SOD activity is that of Kakar et al. The reaction mixture was prepared by adding 0.1 ml of phenazine methosulphate (186 μmol), 1.2 ml of sodium pyrophosphate buffer (0.052 mmol; pH 7.0), 0.3 ml of the supernatant after centrifugation at 1500 × g for 10 min followed by centrifugation at 10,000 × g for 15 min of homogenate. 0.2 ml of NADH, 780 μmol in total. This will initiate the enzyme reaction, but the enzyme reaction will then be stopped after 1 min with 1 ml glacial acetic acid. The amount of chromogen formed was measured by an absorbance at 560 nm. All results are expressed in units/mg protein.(34)

d. Determination of Malondialdehyde (MDA) content

The TBARS assay is the estimation of Malondialdehyde (MDA) concentration to calculate lipid peroxidation levels. The reaction mixture was as modified by Iqbal. It contained 0.58 ml of phosphate buffer, pH 7.4, with a 0.1 mol; it included 0.2 ml of the homogenate sample, 0.2 ml of ascorbic acid with a concentration of 100 mmol, and 0.02 ml of ferric chloride, which also has 100 mmol concentration. The mixture was placed in a shaking water bath at 37°C for an hour. The reaction was put to a halt with 1.0 ml of 10% trichloroacetic acid added to it. 1.0 ml of 0.67% thiobarbituric acid was added after the samples, and tubes were maintained in a boiling water bath for 20 minutes prior to being put into ice. Samples were centrifuged for 10 minutes at a rate of 2500 × g. Concentration of TBARS in each sample was measured through a spectrophotometer with a reagent blank, using the optical density at 535 nm for supernatant(35). The results were expressed as nmol TBARS/min/mg tissue at 37°C using a molar extinction coefficient of 1.56 × 105 M^{-1} cm^{-1}.

2.5 Statistical analysis

The mean ± SEM values for each group were calculated. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Differences between experimental conditions were considered significant at a p-value below 0.05

3. Results

3.1 Total yield from Soxhlet extraction:

The yield of crude extract from dried Murraya leaves using methanol, chloroform and water were found to give a mean ± SD of 3.35± 0.09 (yield 12.92%), 2.18± 0.05 (yield 8.42%) and 2.9± 0.04 (yield 11.82%) from 25 gms. Table 1 shows extract yield exhibited from dried Murraya koenigii leaves. The crude employing the methanolic solvent allowed the total yield of 3.35 grams; this was relatively greater than aqueous extract. On the other hand, the yield obtained from the chloroform extract was relatively low.

3.2 In Vitro Antioxidant ability of Murraya koenigii

3.2.1 Determination using the DPPH assay.

The ability of the DPPH radical to be foraged was observed spectrophotometrically at different concentrations of the extract. The results showed that the highest possible amount of DPPH+, measuring 66.5%, was obtained from the chloroformic extract of Murraya koenigii. On the other hand, the methanolic extract of Murraya koenigii yielded a slightly lower amount of DPPH+, measuring 63.6 % followed by aqueous extract giving 43.2%. The findings of this study indicate that the chloroformic extract had a higher capacity to scavenge DPPH radical cation compared to the other extract.

3.2.2 Determination using the ABTS assay

In the experiment, different concentrations of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were utilized, varying from 0.2 mg/ml to 1 mg/ml. The radical cation ABTS+ was generated using potassium persulfate. The results showed that the highest possible amount of ABTS+, measuring 91.18 %, was obtained from the chloroformic extract of Murraya koenigii. On the other hand, the methanolic

extract of Murraya koenigii yielded a slightly lower amount of ABTS+, measuring 85.49% followed by aqueous extract giving 61.45%. These findings indicate that the chloroformic extract had a higher capacity to generate ABTS+ compared to the other extract. The different concentrations of ABTS used in the experiment influenced the amount of ABTS+ generated (Fig.3)

3.2.3 Antioxidant assessment using The FRAP assay

. The FRAP value for the chloroformic extract was recorded at 37.7%, representing the highest value among the solvents tested, which was followed by methanolic extract with 32.7%. The aqueous extract gave 21.6 %, the least amount of inhibition at 1 mg/ml.

3.2.4 Assay for scavenging lipid peroxidation

The investigation into the oxidation degradation of lipids was conducted utilizing aqueous, methanol, and chloroform extracts of Murraya koenigii. The findings were suggestive of aqueous extract exhibiting a lipid oxidation degradation level of 1.58±0.01, whereas the methanolic extract showed a degradation of 2.59±0.02. In contrast, the chloroform extract exhibited the most significant degradation at 14.07±0.05. These results imply that the chloroform extract possesses a superior ability to inhibit lipid oxidation when compared to both the methanol and aqueous extracts, as evidenced by the greater percentage of degradation recorded. It is likely that the varying concentrations of the extracts employed in the study affected the degree of lipid oxidation degradation observed(Table 5).

3.3 Effect of Murraya koenigii treatment on Brain anti-oxidative status

Table 6 shows changes in brain activities of antioxidant enzymes, and TBARS contents in all the experimental groups of rat. The administration of chloroformic extract from Murraya koenigii (CEMK) resulted in a significant ($p < 0.001$) modification of the concentrations of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and thiobarbituric acid reactive substances (TBARS) in a manner that was dependent on the dosage. The activity of SOD, CAT, and GPx were considerably suppressed in the clozapine-treated group when compared to normal control group and again significantly restored on treatment with CEMK at 100 and 200 mg/kg doses. TBARS was significantly increased in Clozapine-treated group of mice when compared with the control group, and CEMK reduced the level of TBARS. CEMK at 400 and 800 mg/kg bw doses could replenish the antioxidant enzymes furthermore and improve the AO status of brain significantly. The administration of chloroformic extract from Murraya koenigii at doses of 100, 200, 400, and 800 mg/kg notably counteracted the reduction in SOD, CAT, and GPx levels induced by Clozapine, in comparison to rats treated solely with Clozapine (Table 6). Clozapine treatment for 28 days induced lipid peroxidation(LPO) as indicated by significant raise in brain MDA levels compared with control rats. Administration of CEMK (100, 200, 400 and 800 mg/kg) along with Clozapine significantly reversed the extent of LPO compared with Clozapine -treated rats.

Table 1: Yield derived from methanol, chloroform and aqueous solvents of Murraya koenigii

sample quantity taken for Soxhlet extraction (gm)	solvents	yield in gm (Mean ± SD)	yield %
25	methanol	3.35± 0.09	12.94 %
25	chloroform	2.18± 0.05	8.42 %
25	water	2.9± 0.04	11.82 %

Table 2: In- Vitro DPPH of Aqueous, Chloroformic and Methanolic extracts of Murraya koenigii

Concentration (mg/ml)	Aqueous extract of Murraya koenigii (%)	Methanolic extract of Murraya koenigii (%)	Chloroformic extract of Murraya koenigii (%)
0.2	3.89±0.09	20.43±0.57	13.72±0.15
0.4	5.06±0.05	21.63±0.5	21.23±0.5
0.6	15.3±0.08	27.6±0.64	45.3±0.46
0.8	36.7±0.12	37.2±0.56	50.4±0.57
1	43.2±0.12	63.6±0.51	66.5±0.46

Percent inhibition values are expressed in Mean ± SD

Table 3: In- Vitro ABTS assay of Aqueous, Chloroformic and Methanolic extracts of Murraya koenigii

Concentration (mg/ml)	Aqueous extract of Murraya koenigii (%)	Methanolic extract of Murraya koenigii (%)	Chloroformic extract of Murraya koenigii (%)
0.2	22.7±0.07	29±0.5	89.46±0.04
0.4	28.5±0.11	35.76±0.11	89.6±0.05
0.6	39.5±0.09	63.89±0.05	89.86±0.59
0.8	48.1±0.05	79.12±0.05	91.4±0.41
1	61.7±0.3	85.7±0.5	93.6±0.5

Percent inhibition values are expressed in Mean ± SD

Table 4 : In- Vitro FRAP of Aqueous, Chloroformic and Methanolic extracts of Murraya koenigii

Concentration (mg/ml)	Aqueous extract of Murraya koenigii (%)	Methanolic extract of Murraya koenigii (%)	Chloroformic extract of Murraya koenigii (%)
0.2	2.9±0.01	16.23±0.57	3.16±0.10
0.4	5.28±0.05	22.93±0.57	7.3±0.41
0.6	10.37±0.46	29.65±0.25	19.14±0.07
0.8	17.6±0.5	31.67±0.51	31.7±0.52
1	21.6±0.4	32.7±0.11	37.7±0.34

Percent inhibition values are expressed in Mean ± SD

Table 5: In- Vitro LPO assay of Aqueous, Chloroformic and Methanolic extracts of Murraya koenigii

Concentration (mg/ml)	Aqueous extract of Murraya koenigii (%)	Methanolic extract of Murraya koenigii (%)	Chloroformic extract of Murraya koenigii (%)
0.2	0.13±0.02	0.29±0.02	1.73±0.05
0.4	0.28±0.007	1.09±0.03	3.5±0.01
0.6	0.73±0.03	1.89±0.05	5.26±0.05
0.8	1.16±0.02	2.18±0.03	11.09±0.06
1	1.58±0.01	2.59±0.02	14.07±0.05

Percent inhibition values are expressed in Mean ± SD

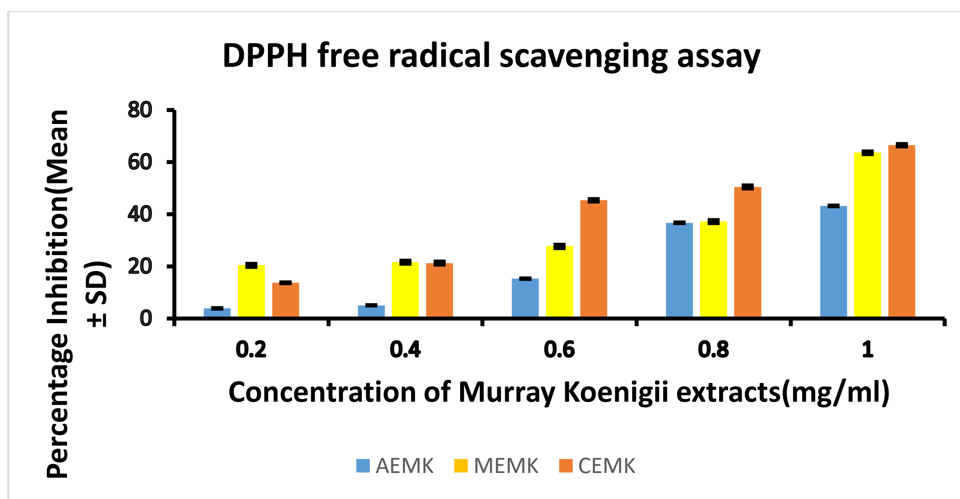

Fig 2: Graphical representation of DPPH radical scavenging activity using aqueous, methanol and Chloroform extract of Murraya koenigii

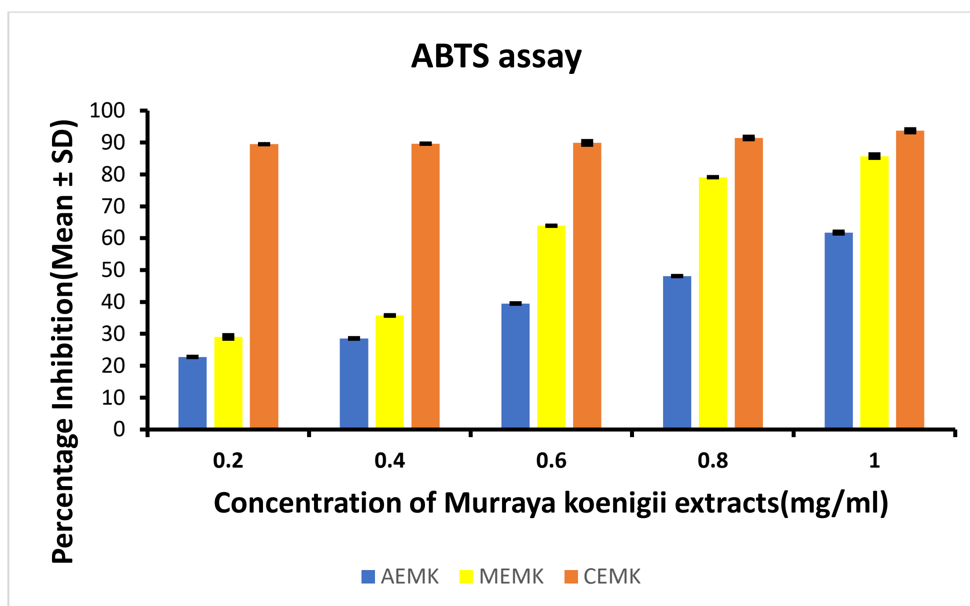

Fig 3: Graphical representation of ABTS radical scavenging activity using aqeous, methanol and Chloroform extract of Murraya koenigii

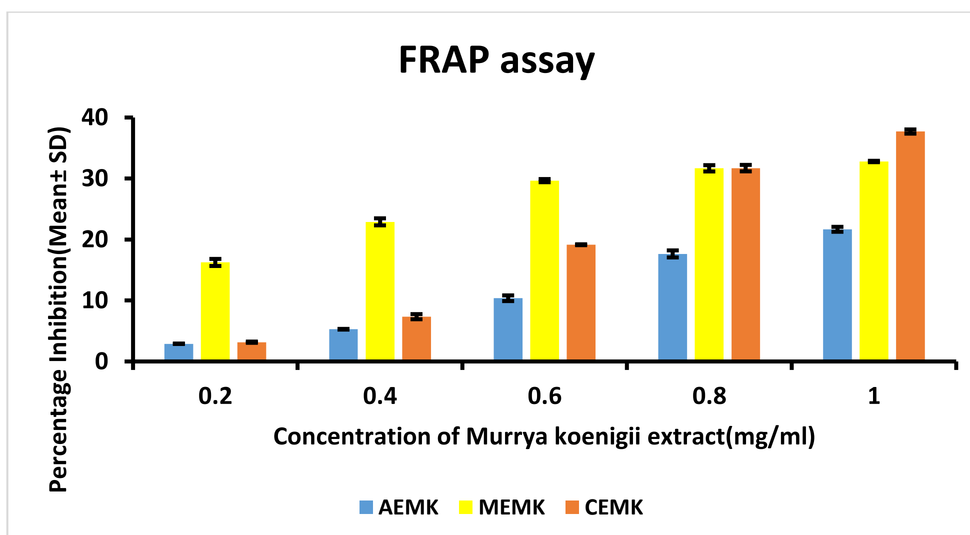

Fig 4: Graphical representation of FRAP assay using Aqueous, Chloroform and methanol extract of Murraya koenigii

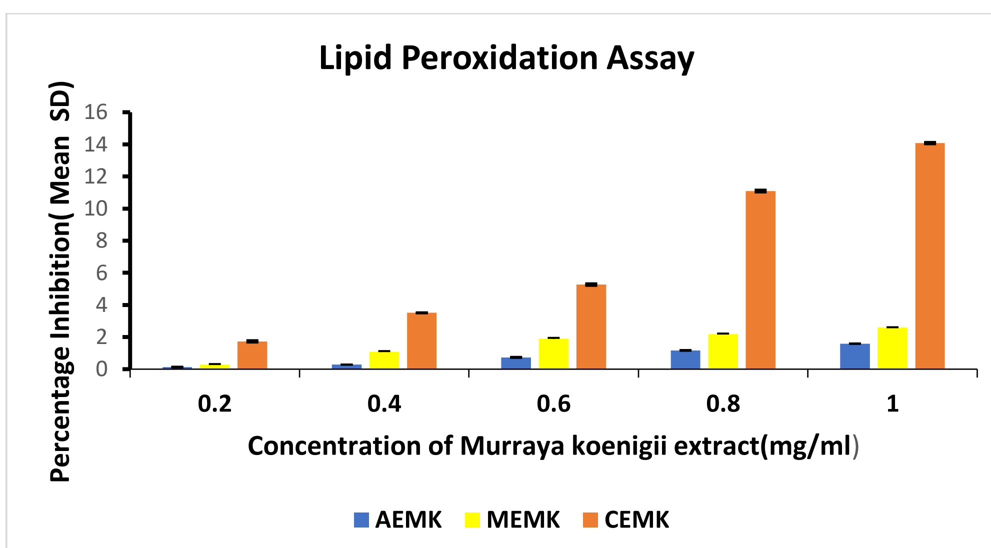

Fig 5: Graphical representation of Lipid peroxidation activity using aqueous, Chloroformic and methanol extract of Murraya koenigii

Groups (n=6)	Dose (mg/kg)	SOD (µ/mg protein)	CAT (µ/mg protein)	GPx (µ/mg protein)	LPO (n moles/mg protein)
Grp I	Normal saline	3.4 ± 0.1	0.28 ± 0.03	8 ± 0.16	5.1 ± 0.09
Grp II	Clozapine (10mg/kg bw)	2.3 ± 0.2*	0.24 ± 0.03*	6 ± 0.47 *	8.3 ± 0.14*
Grp III	Cloz(10)+CEMK(100)	2.7 ± 0.0^{c}	0.28 ± 0.01^{a}	6.4 ± 0.24c	5.1 ± 0.29^{a}
Grp IV	Cloz(10)+CEMK(200)	3.5 ± 0.0^{b}	0.32 ± 0.0^{a}	8.2 ± 0.27^{b}	4.4 ± 0.26^{a}
Grp V	Cloz(10)+CEMK(400)	4.1 ± 0.1^{a}	0.33 ±0.01^{a}	10.1 ± 0.3^{a}	4 ± 0.24^{a}
Grp VI	Cloz(10)+CEMK(800)	4.4 ± 0.4^{a}	0.36 ± 0.0^{a}	11.7 ± 0.4^{a}	3.3 ± 0.23^{a}

Table 6: Effect of Chloroformic extract of Murraya koenigii (CEMK) on Anti-oxidant enzymes. *suggests comparison with Group-I. Treatment groups were compared with Group-II wherein c=p<0.05, b=p<0.01, a=p<0.001. CEMK: Chloroformic extract of Murray koenigii, SOD: Superoxide dismutase, LPO: Lipid peroxidation, CAT: Catalase, GPx: Glutathione peroxidase

4. Discussion

The present study has been carried out using rats for evaluating the beneficial outcome of Murraya koenigii extract on Clozapine induced oxidative stress. Pointing out the underlying oxidative stress mechanisms in clozapine therapy as well as mechanisms by which Murraya koenigii exerts its antioxidative property was an important aim of our study.

Extraction: Methanolic solvent allowed the extraction of highest yields in comparison to aqueous and chloroformic solvents. The selection of a solvent for the extraction of bioactive compounds should prioritize low toxicity, the ability to evaporate at relatively low temperatures, and optimal physiological permeability(37). Additionally, the solvent should not cause dissociation of the extract, should enhance the stability of crude metabolites, and must not facilitate their degradation(38). By employing Soxhlet extraction with different temperatures, retention times, and solvents, the study assesses the effectiveness of these methods in extracting bioactive constituents(39). Preliminary phytochemical investigations of the leaf extracts from Murraya koenigii have revealed the presence of saponins, flavonoids, coumarins, tannins, terpenoids, glycosides, and phenols, as documented in our earlier research(20). This plant exhibits significant variability in its phytochemical composition, indicating that no single solvent can effectively extract all of the compounds present(40).

Invitro Antioxidant capacity: Extracts from Murraya koenigii have demonstrated notable antioxidant activity when evaluated using the DPPH, ABTS, FRAP and Lipid peroxidation assays. The results of our investigation showed that chloroformic extract had the most radical scavenging ability. This in in accordance with Parithy et al. (2021), who demonstrated the superiority of ultrasonic-assisted extraction (UAE) in comparison to microwave-assisted extraction (MAE) and solvent-assisted extraction(SAE), also highlighting the radical scavenging capabilities of curry leaf extracts by their higher inhibition percentages against oxidation (Parithy et al., 2021). Similar results were obtained with acetone extracts exhibiting significant ABTS+ inhibition that correlated with increasing concentrations(41).

In the FRAP assay, the reaction entails the conversion of Fe3+—TPTZ (iron[III]-2,4,6-tripyridyl-S-triazine) to Fe2+—TPTZ through the action of an antioxidant compound. Our results show a pattern for Murraya koenigii, where its reducing power is enhanced with higher dosages. All administered doses demonstrated significantly greater activities compared to the control, reflecting an increased reducing capacity. The literature indicates that the FRAP method is effective in assessing the total antioxidant capacity of fresh biological fluids, including plant homogenates and pharmacological plant products (43). Our findings align with those of Sonter et al. (2021), where the methanolic extract of Murraya koenigii represented the highest scavenging ability as compared to acetone, water, chloroform, and hexane(41). Usually, phenolic compounds extracted from plant infusions display commendable antioxidant characteristics (44).

The investigation into the oxidation degradation of lipids imply that the chloroform extract possesses a superior ability to inhibit lipid oxidation when compared to both the methanol and aqueous extracts, as evidenced by the greater percentage of degradation recorded. Lipid peroxidation of unsaturated fatty acids is commonly employed as an indicator of increased oxidative stress and the resulting cytotoxic consequences(45). The protective effects of these plants are likely due to the presence of tannins, flavonoids, and phenolic compounds, as indicated by the preliminary phytochemical analysis of the chloroform and methanol extracts of the leaves.

Effect of Free radicals: Free radical injury to the central nervous system (CNS) occurs as a result of its significant oxygen consumption, elevated lipid levels, and a deficiency in antioxidant enzymes relative to other tissues. Research has shown that clozapine can promote the oxidation of mitochondrial proteins essential for energy metabolism in both neuroblastoma cells and lymphoblastoid cells obtained from individuals diagnosed with schizophrenia(47). A variety of studies have demonstrated that clozapine

promotes the generation of reactive oxygen species(48,49). In a research investigation involving patients receiving prolonged clozapine therapy, increased concentrations of the antioxidant enzyme superoxide dismutase were observed in red blood cells (50)and rat brain (13,51).

Effect of Murraya koenigii on Brain Antioxidant status: The activities of SOD, CAT, and GPx were markedly reduced in the group treated with clozapine in comparison to the normal control group. However, these activities were significantly restored following treatment with CEMK at all administered doses (Table 6). SOD activity is a sensitive measure of oxidative damage because it scavenges the superoxide anion to generate hydrogen peroxide, reducing harmful consequences. Catalase is an enzyme antioxidant found in all vertebrate tissues. It decomposes hydrogen peroxide and protects the tissue against highly reactive hydroxyl radicals(34). MDA is defined as the most sensitive biomarker of lipid peroxidation, and it is regarded a helpful measure of oxidative stress state(52). GPx plays a crucial role in the cellular antioxidant defense mechanisms by detoxifying peroxides and hydroperoxides, utilizing glutathione as the reducing agent(53).Our results on the oxidative stress parameters reveals that, there was a highly significant increase in MDA levels in CLZ-treated group in comparison to the negative control group. There is no doubt that Oxidative stress and antioxidant system play an important role in pathophysiological cerebral changes as seen during supplementation of *Sonchus asper* in rats improving the activity of antioxidant enzymes, showing protection against free radicals(34).

Our study is in consonance with Patil et al., where ethanolic and aqueous extracts of Murraya koenigii were found to significantly counteract the reduction in forebrain SOD and CAT levels induced by haloperidol, while also markedly decreasing LPO and restoring the diminished GSH levels resulting from chronic haloperidol administration(54). Similar effects were replicated with the use aqueous extract of Murraya koenigii at higher doses in the brain of Paraquat-treated animals(55). Additionally, Murraya leaf extract showed a significant increase in antioxidant parameters and reduced lipid peroxidation in doxorubicin-induced cardiotoxicity in rats, thus establishing its cardioprotective property(56). It has been reported that M. koenigii stimulates various antioxidant defense enzymes(58). Another proposed mechanism of action for M. koenigii involves its ability to scavenge free radicals, particularly as an effective hydroxyl radical (OH−) scavenger (59). The protective influence of M. koenigii against oxidative stress is likely attributed to the presence of carbazole alkaloids, polyphenols, and flavonoids, everything mentioned are recognized for their antioxidant capabilities(61).

Role of Flavanoids in mitigating oxidative stress: Flavonoids are a class of polyphenolic secondary metabolites commonly found in plants and often included in human nutrition. These compounds demonstrate various biological activities, such as anti-inflammatory, anticancer, and anti-Alzheimer properties(62,63). The O-dihydroxy (catechol) arrangement improves flavonoids' radical scavenging properties, establishing this structure as a crucial site for radical interactions(64). These phytochemicals demonstrate their antioxidant properties by neutralizing a range of radicals, which include hydroxyl radicals (. OH), superoxide anion radicals (O2 . –), singlet oxygen (1O2), alkoxyl radicals (RO), peroxyl radicals (ROO), and peroxynitrite (ONOO). Additionally, George and colleagues (65) indicated that the free-radical scavenging abilities of antioxidant compounds are often linked to their capacity to generate stable radicals following their interaction with free radicals as seen in the case of Flavanoids(66,67). Consequently, this study demonstrated that the chloroform extract of Murraya koenigii (L.) Spreng. leaves have the capacity to significantly reverse the elevated levels of oxidative stress markers caused by the antipsychotic drug Clozapine, thereby enhancing the overall antioxidant status of the brain.

5. Conclusion

The protective effects of Murraya koenigii (L.) Spreng. leaf extract may stem from its ability to counteract free radicals, attributed to its antioxidant properties. This indicates that pre-treatment with the extract could enhance the brain's antioxidant enzymes, which are essential for combating oxidative stress caused by clozapine during its antagonistic effects on dopamine, serotonin, and histamine receptors. Nevertheless, the precise molecular mechanisms through which Murraya leaf extract provides its protective benefits against oxidative damage requires further investigation. The high concentrations of polyphenols and flavonoids present in the extract may offer a reasonable explanation for its significant antioxidant capabilities. Consequently, Murraya koenigii (L.) Spreng. leaf extract could serve as an adjunctive therapy alongside clozapine or be considered a viable alternative in the management of neurodegenerative disorders.

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