

Effect of *M. chamomilla* L. tea on chlorpromazine induced catalepsy: A neuroprotective study

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Abstract: We determined anti-Parkinson's activity of *M. chamomilla* L. tea in chlorpromazine (CPZ) developed investigational animal model. In this research, effects of *M. chamomilla* L. tea 2.14ml/ kg P.O were studied on cataleptic behavior and its effect on brain histopathological changes and immunohistochemistry (IHC) in rats. The experimental design was developed by administering CPZ (3mg/kg, I/P) for twenty-one days to produce Parkinson's disease-like symptoms to 4 animal groups. We observed that chlorpromazine significantly produced motor dysfunctions (catalepsy) in a time period of twenty-one days. The *M. chamomilla* L. significantly ($P < 0.005$) minimized/shorten/taper down catalepsy in rats just like standard group (Levodopa/carbidopa treated group). The maximum reduction was observed from both treated and standard groups on the 21st day. *M. chamomilla* L. treated rats mid brain sections showed presence of proliferative blood vessels, increase cellularity with reactive glial cells as compared to CPZ group. Furthermore, immunostaining CD68 & CD21 of *M. chamomilla* L. treated rats mid brain region showed few CD68 cells & no polymorphs neutrophils after CD21 staining. Thus, this research work disclosed the neuroprotective effect of *M. chamomilla* L. tea against Parkinson's disease-like symptoms or anti-Parkinson's activity induced by CPZ.

Keywords: Catalepsy, Chlorpromazine (CPZ), Histopathology, Immunohistochemistry, *M. chamomilla* L., Neuroprotective effect, Parkinson's disease.

INTRODUCTION

The ongoing deterioration of dopamine neurons that innervate the striatum results in Parkinson's disease (Gash *et al.*, 1996). A decline in dopaminergic activity in the striatum of rats such as by intake of neuroleptic drugs or by substantia nigra lesions, leads to symptoms (akinesia, rigidity, tremor) that resemble to those of Parkinson's disease, which is a clinical exemplification of striatal dopamine deficiency syndrome. This state can be rectified by L-DOPA which restores the striatal dopaminergic activity (Stadler, Lloyd, Gadea-Ciria & Bartholini, 1973).

Several epidemiological data has demonstrated that Parkinson disease is more common in geriatric patients and motor symptoms usually arise when the disease process is far advanced as a result of dopaminergic cell destruction and collection of alpha synuclein, a major component of Lewy bodies (Xu & Pu, 2016). Neurodegeneration due to dopaminergic cell death results in free radical formation like ROS (reactive oxygen species) and NOS (nitric oxide synthase) that are further responsible for developing oxidative stress and degeneration (Olanow, 1992). This postulation has brought considerable attraction towards antioxidants, which could play possible neuroprotective role in

managing Parkinson disease. However, current therapies for Parkinson's disease provide symptomatic ease but till date, there is no therapeutic option to end or either slowdown disease advancement. Natural remedies could be considered as alternative or complementary medicine to explore new molecules useful for the management of neurological disorders. Chamomile tea is considered to have good antioxidant potential. Previous studies have demonstrated the antioxidant potential of chamomile in different diseases like in inflammatory disorders, cancer, dyslipidemia, anxiety and depression (Gardiner, 1999). *M. chamomilla* L. contains flavonoids (quercetin, apigenin, luteolin and chrysin), essential oil, volatile oils (alpha-bisabolol), bitter glucosides and coumarine (Zanoli, Avallone & Baraldi, 2000). According to researchers, psychoactive properties of *M. chamomilla* L. are attributable to the existence of flavonoids in chamomile (Kato *et al.*, 2008; McKay & Blumberg, 2006; Zanoli *et al.*, 2000). The dried heads of *M. chamomilla* L. flowers are utilized in folk medicine to concoct sedative-hypnotic and spasmolytic tea (Viola *et al.*, 1995). Flavonoids such as apigenin and chrysin which are derived from *M. chamomilla* L. have been reported as anxiolytic compounds, their mechanism of action could be bridged to activation of the GABA (A) receptor in brain (Avallone *et al.*, 2000; Can, Ozkay, Kıyan, & Demirci, 2012; Zanoli *et al.*, 2000). There are different clinical evidences that confirmed that plant derived flavonoid compounds may

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result in response of any neuronal insult against oxidative damage and limits neuro inflammation (Nikam & Sontakke, 2009). Moreover, these chamomile flavonoids which have a potent inhibitory effect on macrophages prostaglandin E2 levels and can perform the action of selective COX-2 inhibitor (Zargaran *et al.*, 2014). As expression of COX-2 enzyme in neurons is instigated by glutamate and inflammatory cytokines (Pasinetti & Aisen, 1998; Tocco *et al.*, 1997) and that PGE2 levels are elevated in Parkinson disease (Cimino *et al.*, 2008; Naeem *et al.*, 2017). On the other hand oxidative burden also performs a pivotal role in the pathogenesis of Parkinson's diseases by causing neurodegeneration of glial cells (Bais *et al.*, 2015). *M. chamomilla* L. extract has antioxidant potential by free radical capturing activity and has lipid peroxidation property (Kato *et al.*, 2008). Current researches have revealed several clinical uses of chamomile tea such as anti-inflammatory, anti-hypolipidemic, antibacterial, antioxidant, anticancer, antidepressant and anti-parasitic activity that propose its established use in different chronic diseases (Bhaskaran *et al.*, 2010). These studies have postulated that daily administration of antioxidants may slowdown the advancement of Parkinsonism. There is only a few or inconstant report on the defensive role of dietary antioxidants especially on chamomile tea in Parkinson's disease.

Carbidopa, levodopa are the allopathic drugs used for the treatment of Parkinson disease but have major adverse effects and are expensive in comparison to herbal medicines which has either few or minor side effects. The present study is designed to assess the anti cataleptic and neuroprotective potential of chamomile tea in Parkinson's disease model. Chamomile tea can act as safer, better and cheaper herbal alternate of Parkinsonism.

MATERIALS AND METHODS

Assessment of acute toxicity by brine shrimp bioassay

In the experiment the aqueous extract of chamomile tea and reagents used were; sea salt (38g/L of distilled water having pH 7.4), test sample, tray for hatching eggs, micro pipettes, lamp for attraction of larvae, distilled water and methanol. The procedure of Mayer *et al.* (1982) brine shrimps cytotoxicity was followed with slight modifications. The test sample (1g) was dissolved in 150ml of water to prepare a stock solution. In separate vials three concentrations 1000, 100 and 10µg/ml were prepared. Ten shrimp's nauplii were transferred to each vial and adjust the final volume up to 05ml with sea salt water. The experiment was performed in triplicate and incubates at 25±1°C for 24h. Afterwards, the numbers of dead shrimp's nauplii were counted and the percentage was calculated

The LC₅₀ was calculated by the following formula
% deaths = (control-test/control) × 100

Animals

Current study was performed on male Wistar rats (150–250 g) that were procured from the University of Karachi HEJ institute (Pakistan). Male Wistar rats were kept under standard laboratory conditions. All animals were kept in a group of 2 rats per cage and provided with normal rat pellet diet (rat chow) in morning and evening as well as drinking water was available throughout a day. The specifications provided in Helsinki Resolution 1964 were followed to handle animals. The protocol was authorized by the BASR Institutional Animal Ethics Committee, University of Karachi under resolution No.10 (66) and were conducted according to the guidelines of National research Council, 1996 (Clark *et al.*, 1997)

Chemicals

Carbidopa/levodopa was purchased from OBS Pharma (Karachi, Pakistan). Chlorpromazine was purchased from Platinum Pharmaceutical (PVT.) Ltd. All analytical grade chemicals were used and purchased from Rehaan Scientific Enterprises (Karachi, Pakistan). Chamomile tea was obtained from local store of Twinning Company Limited.

Preparation of Chamomile flowers tea extract

For the preparation of chamomile tea extract 1gm of chamomile flowers were mixed in 150 ml (100°C) boiling water and allowed to steep then extract was filtered with the help of Whatman filter paper # 1 (Wang *et al.*, 2005). Extract was freshly made every day and was given 30 min later to chlorpromazine injection. The study duration was twenty one days.

Induction of Parkinsonism

Induction of Parkinsonism has been done by giving 3mg/kg/day chlorpromazine (CPZ) to each Wistar rat for up to twenty one days. CPZ produces pseudo Parkinsonism by blocking dopamine receptors in mid brain which produce motor disturbances. Motor impairments and catatonic behavior can be used as a tool in pharmacological experiments to elaborate Parkinson's animal model (Bais *et al.*, 2015; Costall & Naylor, 1974).

Experimental protocol

All the male Wistar rats were randomly partitioned into 4 groups. Each group was comprised of 10 rats, as follows: in group I, normal control rats received a standard volume of normal saline (control group); in group II, PD-like symptoms were induced with CPZ and the rats received only CPZ (3 mg/kg/day, I/P) daily for 21 days (negative control group); in group III, PD-like symptoms were introduced with CPZ and levodopa/carbidopa was administered at 30mg/kg/day (standard group); in group IV, PD-like symptoms were induced with CPZ and the rats were treated with chamomile tea 2.14 ml/kg/day (test group) respectively for 21 days. CPZ was given intraperitoneally to each rat thirty minutes prior to the

LC₅₀ of chamomile tea

Dose (µg/ml)	No. of Shrimps	No. of Survivors	% Mortality	Standard drug	% Mortality
1000	30	28	6.66%	Etoposide	70 %
100	30	29	3.33%		
10	30	30	0.00%		

Standard Drug: Etoposide, % Mortality: 70%

administration of extract and standard drug (Khatoon *et al.*, 2016). Upon completion of study all rats were sacrificed, their brains were immediately removed, kept in chill 0.9% NaCl and preserved in formalin solution in separate containers.

Catalepsy test

Bar method as reported previously by Khatoon *et al.* (2016) was used to assess cataleptic behaviour of Wistar rats after 14 & 21 days of CPZ administration. In this experiment, both front paws of rats were placed in a half rearing position on a horizontal wooden bar 9 cm elevated from the base of the floor. A cut off time of 720 seconds and three attempts were allowed to each rat to maintain this position. The intensity of catalepsy was estimated as length of time the test subject maintained this posture. When the forepaw touched the floor or when the mouse climbed the bar, this point was considered as end point of catalepsy (Naem *et al.*, 2015).

Histopathological examination

From the whole brain sample, only the mid brain area of each group of treated rats (control, negative control, chamomile & Levodopa/carbidopa) were dissected out in small sections then these sections were taken in petri dish and washed out with cold icy 0.9% saline. Buffered formalin solution was used for fixation of selected sections. About 24 to 40 hour after fixation, alcohol was used for the dehydration of tissues. Now the dehydrated tissues were fixed in wax following the H & E technique (Humason, 1962).

Immunohistochemistry (IHC)

Whole brain tissues were used in all cases. For immunohistochemistry brain tissues of control, negative control, chamomile and Levodopa/carbidopa treated Wistar rats were sectioned with cryostat at a thickness of 12 micro meter. Brain sections were treated with buffers for antigen retrieval and detection of inflammatory cells (leukocytes and macrophages). Then brain sections were procreated with primary antibody (against CD68 and CD21) & DAB (3,4,3',4'-tetra amino biphenyl hydrochloride) (Cell Marque, USA). Now all sections were washed with 0.9% saline. Finally Harri's Haematoxylin was used for counterstaining of all sections. Semi-quantitative grading was done to determine the CNS inflammation under 400 power magnification microscope (Klopfleisch, 2013).

STATISTICAL ANALYSIS

Data was analysed with one-way ANOVA followed by Tukey's post hoc tests, with significance set to $p < 0.005$. All analyses were performed using SPSS version 21. Results obtained were presented as mean $\pm$ standard deviation.

RESULTS**Assessment of acute toxicity by brine shrimp bioassay**

Assessment of acute toxicity analyzed by Brine Shrimp Method revealed that the mortality rate of chamomile tea extract at 1000µg/ml is 6.66%, 100µg/ml is 3.3% while at 10µg/ml is also 0.00%, which is very less as compared to standard drug Etoposide which has mortality rate of 70%. Chamomile tea extract is extremely safe as it showed no cytotoxicity even on higher concentrations.

Effect on Catalepsy

Our results showed that CPZ significantly produced cataleptic behavior in negative control rats as evident by catatonic imposed behavior during bar test. However, chamomile treated Wistar rats significantly ($P < 0.005$) revert the effect of CPZ induced catatonic behavior. One way ANOVA revealed that rats treated with chamomile extract showed improved cataleptic scores after 14 days of dosing ($p < 0.01$) in comparison to negative control and similar to standard group ($p > 0.05$) respectively.

However the significance was gradually increased after 21 days of treatment as chamomile treated rats showed normal behavior just like control group and scored reduced catatonic behavior as compared to negative control group ($p < 0.005$).

Histopathological findings

Histopathological slides of negative control group (CPZ) showed presence of gliosis, distortion & broken fragmented neurons which shows necrosis of neurons in the mid brain region as compared to normal group (fig. 2a and 2b). (fig. 2b) CPZ treated group showed edema, infiltration along with proliferative vessel as compared to chamomile and L-dopa/carbidopa treated rats (fig. 2c and 2d respectively). Chamomile 21-day's treatment showed improved and regenerative neuronal cell picture as standard group which represents, proliferative blood vessels, reduced edema, presence of astrocytes (fig. 2c).

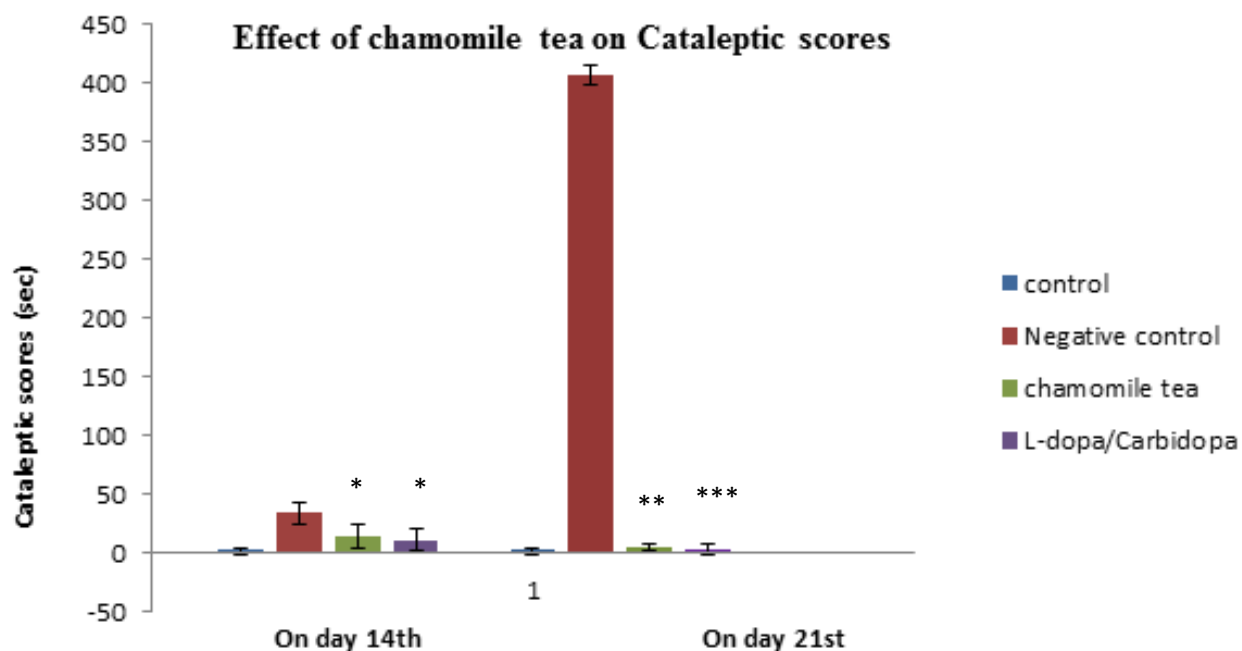


Fig. 1: Graph representing effect of chamomile tea on catalepsy score
Values are presented as $\pm$ SD * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ in comparison to negative control group (chlorpromazine treated)

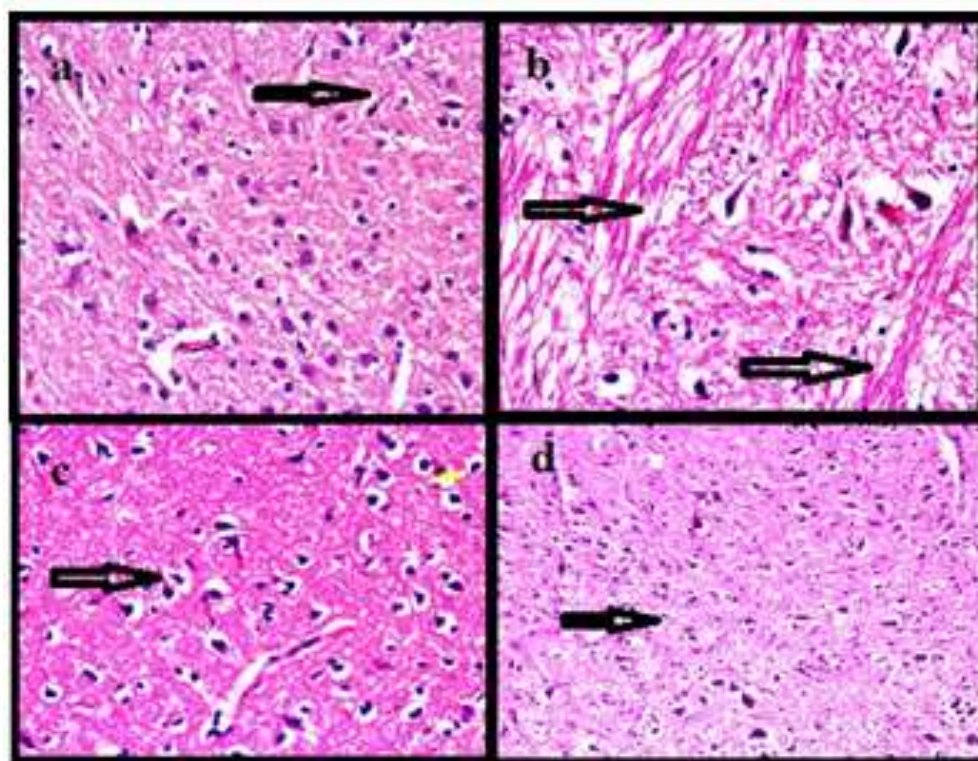


Fig. 2: Histopathological slides

a) Microphotographs of H & E at x400 representative section of control showing normal neuron (shown by arrows) lying in fibrillary background composed of neuroglial cells. b) CPZ treated rat's brain showed presence of large to medium sized neurons with fibrillary background showing eosinophilic necrosis of neurons in the background of oedema along with proliferative vessels. c) Representative section of chamomile treated mid brain showing increased proliferative blood vessels and increased cellularity with presence of reactive astrocytes. d) Representative section of levodopa treated animal's brain showing relatively normal looking medium sized neurons along with proliferative blood vessels, increased cellularity and reactive glial cells.

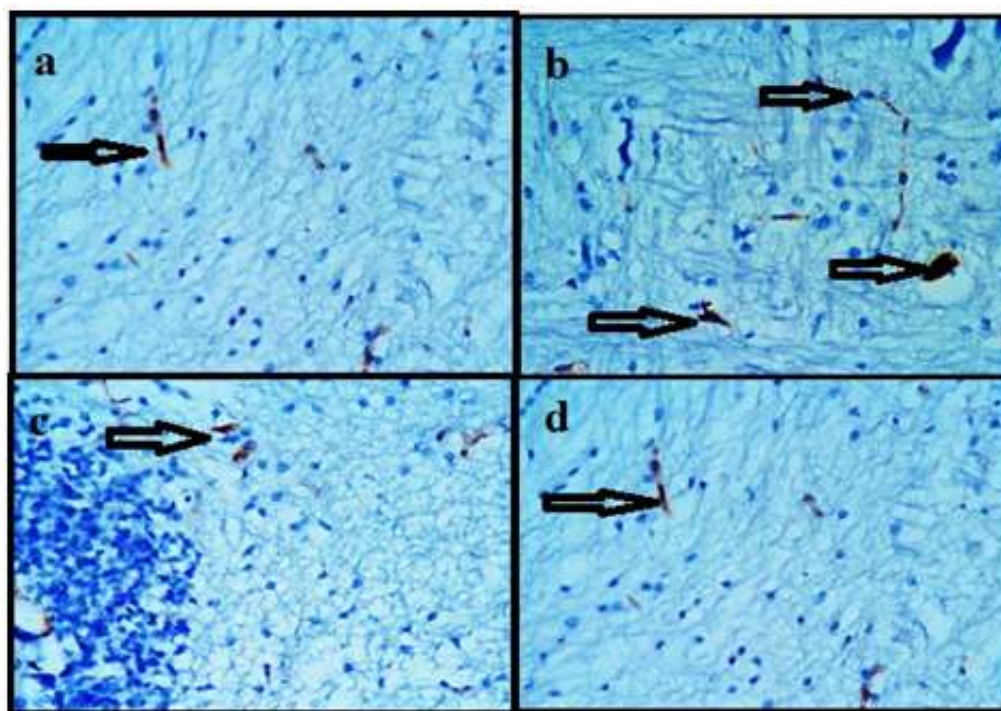


Fig. 3: Results of immunostaining (CD 68)

a) CD68 Immunostaining of control rat's brain Microphotograph power x400 represents no to mild macrophages infiltration after CD68 staining. b) CPZ treated rat's brain showed presence of moderated macrophages after CD68 staining. c) Representative section of chamomile treated animals showed few macrophages after CD68 staining d) Representative section of levodopa treated animals showing infiltration of few CD68 positive cells.

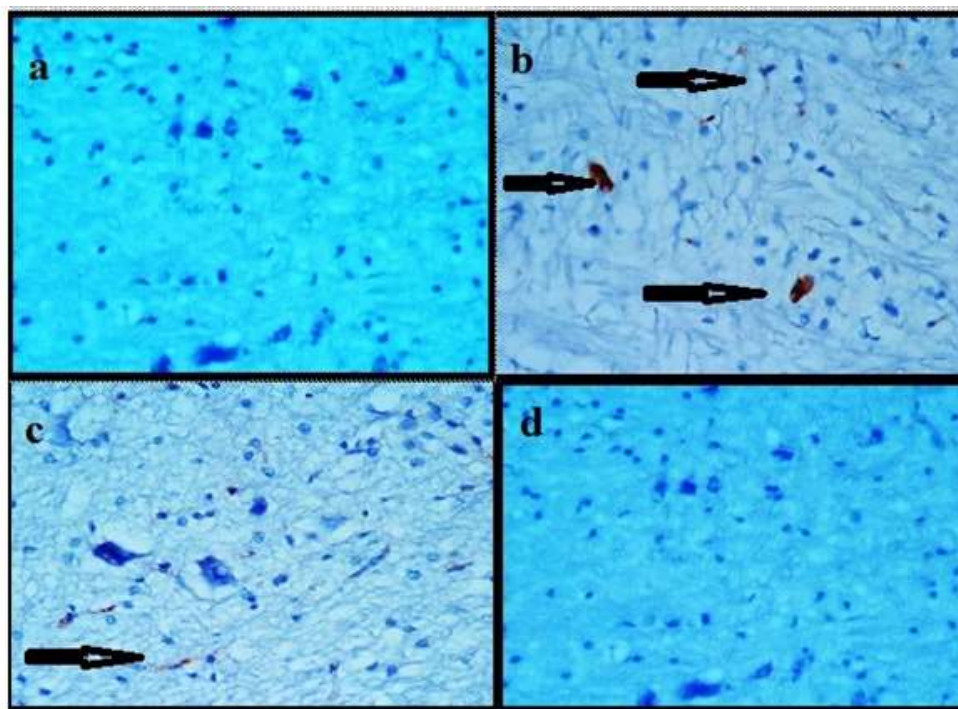


Fig. 4: Result of immunostaining (CD 21)

a) CD21 Immunostaining of control rat's brain Microphotograph power x400 represents no to mild neutrophil infiltration after CD21 staining. b) CPZ treated rat's showed presence of moderate neutrophils after CD21 staining. c) Representative section of chamomile treated animal showed few neutrophils after CD21 staining. d) Representative sections of levodopa treated animal showing infiltration of few CD21 positive cells

Immunostaining findings

Immunostaining of control rats mid brain section showed mild macrophages and neutrophils infiltration after CD68 and CD21 staining respectively (figs. 3a and 4a), whereas CPZ treatment caused moderate macrophages and polymorphs neutrophils after CD68 and CD21 staining which shows microglial inflammation as shown in fig. 3b and 4b). However, sections examined after chamomile treatment (fig. 3c and 4c) show no macrophages & polymorphs neutrophils after CD68 and CD21 staining just like levodopa/carbidopa treated group, as shown in figs. 3d and 4d.).

DISCUSSION

Current study describes the neuroprotective role of chamomile flower tea extract in CPZ induced Parkinson's model. Inflammation of neurons is a main hallmark of many advanced neurodegenerative diseases like Parkinson's disease. Several in-vitro and in vivo researches suggest that microglial activation and its derived prostaglandins and other cytokines perform a crucial role in the disease progression of Parkinson thus, inhibition of inflammatory cascades is an effective therapeutic strategy to limit the progression of PD (Dragicevic *et al.*, 2015). In the present study we have used CPZ to induce Parkinson's like symptoms for the induction of Parkinsonism in experimental animals by neuroleptics like haloperidol and CPZ is widely known for it (Krishna *et al.*, 2008). A strong direct correlation between motor destruction and DA deprivation in neuroleptic produced mouse model of PD was previously documented as well (Saeed *et al.*, 2017). It has been studied that the dopamine receptors which are confined on striatal neurons are responsible for producing cataleptic behavior (Sanberg, 1980). The administration of CPZ is responsible for increased formation of free radicals which produces oxidative stress in the mid brain region (Sandhu & Rana, 2013). The decreased glutathione and superoxide dismutase levels were perceived in different Parkinson's experimental models induced by CPZ (Naeem *et al.*, 2015; Rasheed *et al.*, 2010). Decreased antioxidants in brain tissues may reduce regenerative mechanism and cause necrosis and hypoxia which ultimately leads towards apoptosis (Lalkovičová & Danielisová, 2016).

Dopamine receptors in the mid brain region especially in striatum are responsible for neuroleptic induced cataleptic behavior (Sandhu & Rana, 2013). Our negative control animals showed immobility and severe cataleptic behavior after 21 days of treatment which is supported by Sanberg (1980) who discovered that CPZ antagonizes dopamine receptor in mid brain on striatal neurons & produces Parkinson like behavior. Further research studies also demonstrated that CPZ induces catalepsy and Parkinson like behavior due to increased oxidative stress (Sandhu & Rana, 2013). The increased oxidative burden

is attributable to the over production of ROS & NOS species in the CNS tissues along with marked reduction in levels of SOD and glutathione (Aragno *et al.*, 1997). Increased superoxide species production in brain leads to activation of cytokines and inflammatory markers and ultimately produces neuro inflammation (Barcia, 2013). In this study *M. chamomilla* L. tea treated animals showed highly significant reduction in cataleptic behavior after 21 days treatment as compared to negative control which showed that chamomile tea extract has potential anti-oxidant activity (Campbell *et al.*, 2004). Earlier studies have showed the antioxidant components of chamomile tea, like luteolin, apigenin, quercetin, chrysin and coumarin as well as their varied therapeutic activities (Srivastava *et al.*, 2010). In one of these studies chamomile tea showed antioxidant activity by increasing antioxidant enzymes and quenching the free radicals (Yoo *et al.*, 2008). Previously, Luteolin and apigenin had shown to exhibit strong antioxidant potential that captures free radicals (Skerget *et al.*, 2005). Flavonoids, phenolic compounds like luteolin, quercetin, chlorogenic acid are the main constituents of chamomile tea which are the potential antioxidants responsible for prevention in cancers, inflammation, aging and neurodegenerative disorders (Ames *et al.*, 1993; Horvathova *et al.*, 2005). Menghini *et al.* (2016) found that chamomile extract successfully reduces the production of NF- κ B, TNF α , PGE2 and cytokines after inflammatory signal in rats (Menghini *et al.*, 2016). This finding indicates that the neuroprotective activity of chamomile tea is not only attributable to its antioxidant property but also due to its anti-inflammatory effect which is also supported by the histopathological and immunostaining results. Chamomile treated mid brain histopathological sections showed increased proliferative blood vessels, increased cellularity and some reactive astrocytes as compared to CPZ treated rats. Our findings are in accordance with recent researches which explain that chamomile contains luteolin and quercetin that possess potent anti-inflammatory, antioxidant, CNS protective, anxiolytic & anticancer properties (Shaikh *et al.*, 2013; Theoharides & Zhang, 2011). eNOS expressed in brain is one of the nitric oxide derived from three isoforms of NOS. It maintains brain microcirculation, prevent leukocyte adhesion, migration and cytokine production (Garry *et al.*, 2015). Quercetin & luteolin increase eNOS levels in substantia nigra and other brain tissues and limits iNOS levels that requires for indirect antioxidant effect (Birman *et al.*, 2012). According to Choudhary *et al.* (2011), glutamate decarboxylase is an enzyme important for the synthesis of Gamma aminobutyric acid (GABA) in brain peripheral neurons. Autoantibodies to glutamate decarboxylase have been associated to a wide range of neurologic conditions, including epilepsy, dementia and migraine (Choudhary *et al.*, 2011). Viola *et al.* (1995) documented that apigenin is a ligand for the central BDZ receptors thus producing anxiolytic and slight sedative effect (Viola *et al.*, 1995).

Nassiri *et al.* (2013) reported that GABA receptors in brain are regulated through quercetin that antagonizes NMDA receptor by decreasing oxidative stress thus decreasing neurodegenerative process. Polarization of macrophage is highly recognized as an essential pathogenic factor in both neoplastic and inflammatory disorders. CPZ-induced neuronal damage activates microglia to produce cytokines which triggers natural defensive mechanism and activation of CD21 & CD68 cells (Barros *et al.*, 2013). Immunostaining of the chamomile treated rat's brain revealed decreased number of inflammatory markers such as macrophages and neutrophils (CD21 and CD68).

The brine shrimp lethality bioassay was also performed to screen the toxicity of crude chamomile tea extract towards brine shrimps, which could also provide an indication of possible cytotoxic properties of the test materials (Apu *et al.*, 2010). Nauplii when exposed to crude extract of chamomile tea showed better survival and swim normally as compared to etoposide. This test suggest chamomile is safe with low or no toxic effects. Furthermore it has been established that our finding suggests that chamomile tea extract may be considered as a potential candidate for the prevention or management of PD. Still, there is a room for further investigational workup at molecular level to elucidate its exact mechanism of action. Hence, on the basis of previous and current research work it can be suggested that regular use of Chamomile tea can be useful and protective against these neurological disorders.

CONCLUSION

Progressive reduction in cataleptic behavior following chamomile tea concluded that it protected dopaminergic cell death from CPZ-developed oxidative burden in rats. The neuroprotective effects of chamomile tea could be due to its antioxidant potential and inhibition of inflammatory cytokines. Suppression of immunohistochemical markers like CD68 and CD21 associated with chamomile tea treatment has proved its anti-inflammatory role in neuroprotection. These findings suggest that chamomile could be valuable as a novel therapeutic molecule for neurodegenerative diseases that are linked to oxidative damage, including PD.

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