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Effect of d-Limonene on behavior and brain oxidative stress in cerebral-ischemia reperfusion injury of mice

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ABSTRACT

The present study explored the neuroprotective role of d-Limonene in the cerebral ischemia-reperfusion injury in mice. Swiss Albino mice (either sex), weighing between 20-30 g were divided in different groups (n≥6). The animals were anaesthetized using Chloral hydrate (400 mg/kg; *i.p.*) and cerebral blood supply was occluded for 10 min and afterwards reperfusion for 24 h. d-Limonene has been administered in 3 doses (50, 100 and 200 mg/kg; *p.o.*) successively to mice for 30 days before surgery. Edaravone (3 mg/kg, *i.p.*) was used as standard drug. The effect of surgery on memory, anxiety, muscle relaxant and locomotor was estimated by using passive avoidance paradigm, elevated zero maze, rota rod apparatus, and actophotometer. After behavioural evaluation, the animals were sacrificed and brains were isolated, homogenized and centrifuged for TBARS, GSH, catalase and brain nitrite estimations. Ishemia-reperfusion injury caused decrease in locomotor, motor-co-ordination, decreased the time spent in open arm, no. of entries and increased in latency time in elevated zero maze and memory of mice. Ischemic mice showed higher brain TBARS and nitrite levels and lower GSH and catalase levels. However, d-Limonene administration significantly attenuated ($p<0.05$) the behavioural and biochemical alterations produced by ischemia-reperfusion injury. d-Limonene treated mice showed a significant ($p<0.05$) decrease in TBARS level and brain nitrite levels. Therefore, d-Limonene may prove to be a beneficial agent in the prevention of stroke.

Keywords: d-Limonene, ischemia-reperfusion injury, bilateral carotid artery occlusion, oxidative stress.

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INTRODUCTION

INTRODUCTION

Stroke refers to a local interruption of blood flow to the brain and is the leading cause of long-term disability, and second leading cause of mortality[1, 2]. Stroke, also known as cerebrovascular accident (CVA), cerebrovascular insult (CVI), or brain attack, is when poor blood flow to the brain results in cell death. Among the neurological diseases, stroke accounts for the largest number of hospitalizations[3]. Stroke can be of 2 types: ischemic and hemorrhagic. Ischemic stroke is characterized by the occlusion of blood vessels by the formation of an obstructive thrombus or embolus in the brain consequently resulting in an inadequate supply of blood and oxygen to the brain, whereas hemorrhagic stroke is characterized by rupture of blood vessels either in the brain or on its surface[4]. About 60-70% of all stroke victims suffer an ischemic stroke[5, 6], whereas, the incidence of hemorrhagic stroke, including subarachnoid hemorrhage and intracerebral hemorrhage, is about 20-25%.

Ischemia is defined as diminution of cerebral blood flow (CBF) to a critical threshold that propagates brain damage involving the entire brain or a selective region[7]. Ischemic stroke follows three main mechanisms: (1) thrombosis, (2) embolism and (3) global ischemia stroke. In Cerebral ischemic stroke, neuronal cell death is caused by a serial pathophysiological event, so called 'ischemic cascade' like energy failure, excitotoxicity, oxidative stress, inflammation, apoptosis etc. These all damaging factors are triggered by decreased/blocked blood flow[8, 9]. Cerebral ischemia experimental models are characterized as global, focal, and multifocal ischemia. In global cerebral ischemia, there is no CBF to any area of the brain, which causes neuronal injury to selectively vulnerable brain areas. Clearly, if global ischemia continued indefinitely, all neurons would die. Global ischemia occurs when cerebral blood flow (CBF) is reduced throughout most or all of the brain, whereas focal ischemia is represented by a reduction in blood flow to a very distinct, specific brain region. In multifocal ischemia, there is a patchy pattern of reduced CBF [10].

A variety of mechanisms are involved in ischemic brain injury. Blockage of a cerebral artery results in interruption of the blood flow and supply of nutrients, glucose and oxygen to the brain. The energy needs of the brain are supplied by metabolism of glucose and oxygen for the phosphorylation of ADP to ATP. Most of the ATP generated in the brain is utilized to maintain intracellular homeostasis and transmembrane ion gradients of sodium, potassium, and calcium. Due to reduction in CBF various substrates, particularly oxygen and glucose that causes accumulation of lactate via anaerobic glycolysis results in collapse of ion gradients, and excessive

release of neurotransmitters such as dopamine and glutamate, activation of phospholipase/ sphingomyelinases[11], release of second messenger ArAc and ceramide[12, 8] , release of cytokines, free radicals, and platelet activation[13] and subsequent receptor activation, leading to calcium influx, metabolic and electrophysiological dysfunction, lipid peroxidation, and other oxidative events. Furthermore, reactive oxygen species, including the superoxide anion, hydroxyl radical, and peroxynitrite radical, have been implicated in neuronal cell damage and death after cerebral ischemia [14] and ultimately, leading to neuronal death and development of an infraction.

Re-establishment of blood circulation to the ischemic brain as soon as possible is the most effective therapy for patients with ischemic stroke. During reperfusion after ischemia, restoration of oxygen and glucose supply reinstates the oxidative phosphorylation that helps to normalize energy demanding physiologic processes, a parallel cascade of deleterious biochemical processes can be triggered that may paradoxically antagonize the beneficial effect of reperfusion[15,16]. Despite the unequivocal benefit of reperfusion of blood to an ischemic tissue, reperfusion itself can elicit a cascade of adverse reactions that paradoxically injure tissue[17]. However, reperfusion itself has a latent risk, as it can cause further damage to brain tissue, such as hemorrhagic transformation, cerebral edema, blood-brain barrier (BBB) leakage and neuronal death[18, 19]. This phenomenon is termed cerebral ischemia/reperfusion (I-R) injury, which is accompanied by a cascade of mechanisms, including glutamate excitotoxicity, calcium overload, oxidative stress, inflammation and apoptosis, eventually leading to cell death[20, 21].

d-limonene (1-methyl-4-(1-methylethenyl) cyclohexane is a natural monocyclic monoterpene and non-nutritive dietary component with a lemon-like odour that has been used in indigenous systems of medicine against various diseases and is a major constituent in the essential oils of citrus fruits, cherry, mint, orange, lemon, mandarin and grapefruit[22]. d-limonene accumulates at huge levels in mature oranges, representing more than 95% of total terpene compounds found in the oil glands from their fruits peel, peel of citrus, and it is produced at a very high metabolic cost[23]. It comprises 90–95% orange peel oil and 75% of lemon peel oil. d-Limonene has demonstrated strong chemo preventive effects in rodent lymphomas[24] and mammary[25], gastric[26], skin, liver, and lung cancers[27]. In humans, consumption of citrus peels has been shown to be significantly related to lower incidence of squamous cell carcinoma, suggesting a protective effect[28]. Investigations of the in vivo disposition of d-limonene and its metabolites are limited. d-Limonene is found to effective as antioxidants,[29] antidiabetic,[30] anticancer,[29] antifungal,[31] antiinflammatory,[32] antistress,[33] GERD,[34] dissolve cholesterol containing gallstones.[35] However, no sufficient studies have been carried out to explore the role of d-Limonene in the treatment of Ischemia-Reperfusion Injury, to

best of our knowledge. Therefore, the present study aims at Investigation for the role of d-Limonene on behavioral and oxidative stress level using cerebral-ischemia reperfusion injury in mice.

MATERIALS AND METHOD

Experiment animal

Adult Swiss mice (either sex), weighing between 20-30 g, were procured from CPCSEA registered approved breeder. The animals were kept in quarantine section till monitoring of health status of animals and subsequently transferred to the housing area. Animals were housed in polypropylene cages with dust free rice husk as a bedding material and maintained under standard laboratory conditions with controlled temperature (23 ± 2°C), humidity (40 ± 10%) and natural (12 h each) light-dark cycle. The animals were fed with standard rodent pellet diet (Ashirwad Industries, Mohali) and water *ad libitum*. The experiment was carried out between 09:00 and 18:00 h. The care of laboratory animals was done following the guidelines of CPCSEA, Ministry of Forests & Environment, Government of India.

Drug and Chemicals

d-Limonene was purchased from Himedia laboratories, Mumbai, edaravone (Edastar) was purchased from Lupin pvt Ltd. DTNB, thiobarbituric acid, tris buffer, reduced glutathione, sodium potassium tartarate, bovine serum albumin was purchased was from Himedia laboratories, Mumbai. Trichloroacetic acid, sodium nitrite, Folin's phenol reagent, sodium hydroxide, copper sulphate was purchased from Loba chemie. Triphenyl tetrazolium chloride was procured from Sisco research laboratories.

Induction of Cerebral Ischemia-reperfusion injury: Global cerebral ischemia was induced using bilateral common carotid artery occlusion. Mice were anaesthetized with chloral hydrate (400 mg/kg, i.p). A midline incision was made in the region between neck and sternum and trachea was exposed. Both the right and left common carotid arteries were located lateral to sternocleidomastoid, freed from the surrounding tissues and vagus nerve was separated. Cerebral ischemia was induced by passing a cotton thread below each carotid artery and the ends of thread were tied tightly with a tape to induce global cerebral ischemia. After 10 min of global cerebral ischemia, the tape on the thread was removed from both the arteries to allow the reflow of the blood through carotid arteries. The incision was sutured back in layers with surgical suture to allow the reflow of blood through carotid arteries. The sutured area was cleaned with normal saline and sprayed with Povidone-iodine antiseptic powder. After completion of surgical procedure, the

animals were shifted individually to their home cage and were allowed to recover. While performing the surgical procedure, the body temperature was maintained at 37^{o}C by heated surgical pad. All the surgical instruments used in the surgical procedure were sterilized prior to use[10], [36]. The surgical/sham control group mice were subjected to the same surgical procedure, but the BCA was not occluded.

Experimental design

Animals were divided into 7 different groups and each group consists of 6 animals. Animals were kept on normal diet and water. Animals were treated with d-limonene/vehicle/standard drug for 30 days. Then on the 30th day animals were undergone ischemia-reperfusion injury surgery, in which both common carotid arteries were exposed over a midline incision and ligated with a thread. Blood supply to the brain was occluded for 10 minutes. After that, the blood flow to the brain was reperfused and incision is sutured and animals were returned to their home cages for 24 h. After 24 h, the animals were exposed to Elevated zero maze, Rota-rod, Actophotometer, Passive avoidance paradigm for behavioral estimations. Afterward, animals were sacrificed for the ttc staining[37] and biochemical estimations such as TBARS[38], GSH[39], Catalase[40], Estimation of Nitrites[41], and brain protein[42].

S.no	Groups (n=6)	Treatment
1.	Normal	Normal group (Saline)
2.	Surgical/Sham control	Surgical control
3.	I-R Injury	Ischemia reperfusion injury
4.	d-Limonene 50	d-limonene 50 mg/kg (oral) + Ischemia reperfusion injury
5.	d-Limonene 100	d-limonene 100 mg/kg + Ischemia reperfusion injury
6.	d-Limonene 200	d-limonene 200 mg/kg + Ischemia reperfusion injury
7.	Edaravone	Standard drug (*i.p.*) + Ischemia reperfusion injury

NEUROBEHAVIORAL TESTS:

Actophotometer[43]

The loco-motor activity was recorded using actophotometer for a period of 5 min. Ambulatory activities were recorded and the loco-motor activity was expressed in terms of total photo beam counts for 5 minutes per animal.

Rota-Rod performance test[44]

The loss of muscle-grip strength is an indication of muscle relaxation. This effect can be easily studied in animals using inclined plane or rotating rods. The difference in the fall off time from rotating rod between control and treated animals is taken as an index of muscle relaxation. Mice were subjected to motor function evaluation by placing them individually on Rota rod, which was

adjusted to the speed of 25 rpm. The fall-off time was recorded for each mouse and the longest period any animal was kept on the rod was 300 s.

Elevated Zero maze test[45]

The elevated zero maze (EZM) is a sensitive behavioral test that reveals animal neophobia or anxiety and can be used to unveil anti-neophobic and anxiolytic actions of drugs. This maze is an elevated (40 cm) black, annular having outer diameter of 45 cm and inner diameter of 30 cm. The runway ring where the mouse can explore is of 6 cm width, which is divided into 4 quadrants, 2 opposing "open" quadrants without walls and 2 opposing "closed" quadrants having 12 cm high walls. The open quadrants have a ridge of 2-3 mm to prevent the mouse to fall off. The walls have thickness of 0.75 cm. Animals were individually placed in closed arm facing towards the open arm and the following parameters were noted for a period of five minutes:

(A). Latency to enter the open arm (LEO):

Latency is the time gap between the first entry of animal in open arm after placing it in the closed arm and signifies the behaviour of animal. In the condition of anxiety the latency time increases significantly as compared to normal animals.

(B). Time spent in open arm (TSO):

Average time spent in open arm by the animal indicates the anxiety level. Lower the anxiety level of animal, more the time animal spent in open arm.

(C). Total number of entries in the open arm (NEO):

The frequency of entry of animal in the open arm indicates the behaviour of animal. Higher the frequency of entry in open arm lower is the level of anxiety.

Passive Avoidance Paradigm[46]

Passive avoidance behavior based on negative reinforcement is used to examine long-term memory. The apparatus consisted of a box (27 cm × 27 cm × 27 cm) having three walls of wood and one wall of Plexiglas, featuring a grid floor (made up of 3 mm stainless steel rods set 8 mm apart), with a wooden platform (10 cm × 7 cm × 1.7 cm), in the center of the grid floor. The box was illuminated with a 15W bulb during the experimental period. Electric shock (20V, A.C.) was delivered to the grid floor. Training (on the 30 day of d-Limonene administration) was carried out in two similar sessions. Each mouse was gently placed on the wooden platform set in the centre of the grid floor. When the mouse stepped-down, placing all its paws on the grid floor, shocks were delivered for 15 seconds and the step-down latency (SDL), which was defined as the time (in seconds) taken by the mouse to step down from the wooden platform to the grid floor, with all its paws on the grid floor, was recorded. Animals showing SDL in the range of 2 – 15 seconds during

the first test were used for the second session and the retention test. The second-session was carried out 90 minutes after the first test. During the second session, if the animals stepped down before 60 seconds, electric shocks were delivered once again for 15 seconds. During the second test, the animals were removed from the shock-free zone if they did not step down for a period of 60 seconds and were subjected to the retention test. Retention (memory) was tested after 24 h (i.e., on the 31st day) in a similar manner, except that the electric shocks were not applied to the grid floor, observing an upper cutoff time of 300 seconds. A significant increase in SDL value indicated improvement in memory.

Statistical Analysis

All the results are expressed as Mean ± SEM. The data of all the groups were analyzed by one way ANOVA followed by Tukey's test using software Graph Pad Prism 6 (Graph Pad Software Inc., USA). A value of $P<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Effect of d-Limonene on step down latency (SDL) using Passive avoidance paradigm

Surgical/Sham control animals showed significant reduction ($p<0.05$) in SDL as compared to the normal group. The mice of I-R injury group showed reduction ($p<0.001$) in SDL as compared to the surgical/sham control group, which shows successful induction of cerebral ischemia-reperfusion injury. Administration of d-Limonene (50, 100 and 200 mg/kg; *p.o.*) and edaravone for 30 consecutive days had significantly prevented the reduction ($p<0.05$) in SDL as compared to I-R injury group.

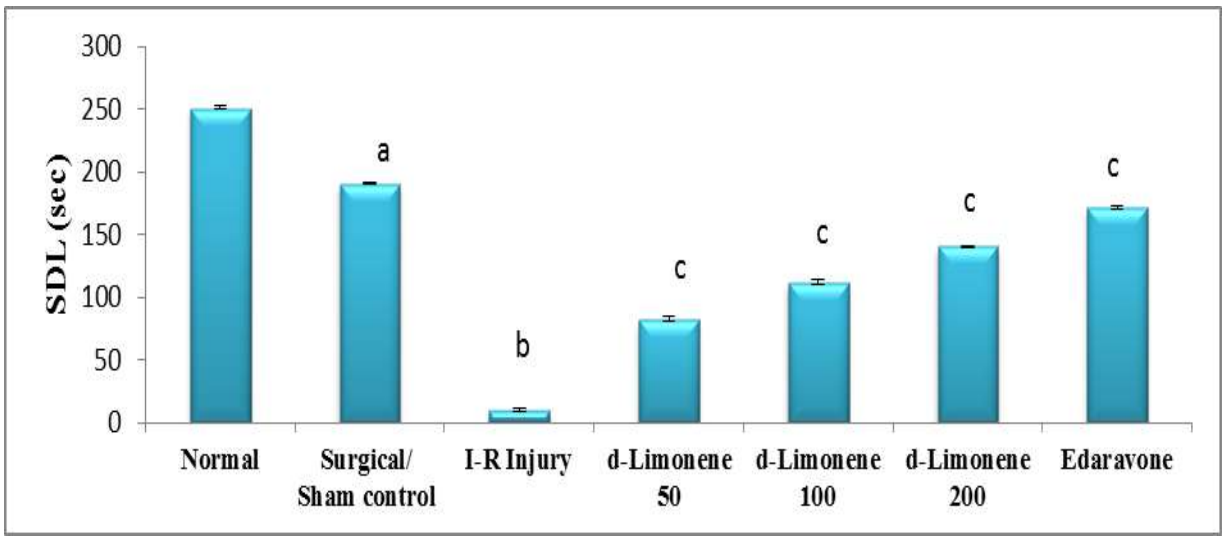

Figure 1: Effect of d-Limonene on SDL using the Passive avoidance paradigm

Values are expressed as mean ± S.E.M. [a]denotes $p<0.05$ compared to normal group, [b] denotes $p<0.05$ compared to surgical/sham control group, [c] denotes $p<0.05$ compared to I-R injury group (one way ANOVA followed by Tukey's test).

Effect of d-Limonene on EZM parameters

Surgical/Sham control group animals showed increase ($p<0.05$) in LEO and decrease in NEO and TSO as compared to the normal group. I-R injury animals showed the increase in ($p<0.05$) LEO and decrease in NEO and TSO as compared to surgical/sham control group, which indicates the successful induction of cerebral-ischemia reperfusion injury. The administration of d-Limonene (50, 100 and 200 mg/kg;*p.o.*) and edaravone for 30 days, successfully prevented the enhancement of LEO significantly ($p<0.05$) and prevented the reduction in NEO and TSO significantly as compared to I-R injury group.

Table 1: Effect of d-Limonene on LEO, NEO and TSO in EZM

Groups	LEO (sec)	NEO	TSO (sec)
Normal	201.3±0.51	17.1±0.75	81.6±0.63
Surgical/Sham control	218.6±0.81[a]	14±2.36[a]	61.6±1.21[a]
I-R injury	241.3±2.06[b]	4±1.41[b]	10.8±0.75[b]
d-Limonene 50	231±1.78[c]	7±0.63[c]	21±0.89[c]
d-Limonene 100	222±1.89[c]	10.3±1.03[c]	32.1±2.13[c]
d-Limonene 200	210±2.52 [c]	12±1.09[c]	41.8±1.32[c]
Edaravone	206±1.41 [c]	13.1±2.52 [c]	54.8±2.04[c]

Values are expressed as mean ± S.E.M. [a]denotes $p<0.05$ compared to normal group, [b] denotes $p<0.05$ compared to surgical/sham control group, [c] denotes $p<0.05$ compared to I-R injury group (one way ANOVA followed by Tukey's test).

Effect of d-Limonene on Fall off Time (FOT) and Loco-motor activity

Surgical/Sham control animals spent less time on the revolving rod and showed less loco-motor activity ($p<0.05$) as compared to the normal animals. I-R injury animals took even less time on the rotating rod and significantly showed less loco-motor activity ($p<0.05$) as compared to surgical/sham control group, which indicates poor motor coordination and muscle weakness due to successful induction of cerebral-ischemia reperfusion injury. However, pretreatment of mice with d-Limonene (50, 100 and 200 mg/kg; *p.o.*) and edaravone for 30 consecutive days showed higher ($p<0.05$) FOT and increase in loco-motor activity as compared to I-R injury animals in a dose dependent manner.

Table 2: Effect of d-Limonene on Fall off Time (FOT) and Loco-motor activity

Groups	FOT (sec)	Locomotor score
Normal	80.6±1.5	153.5±1.37
Surgical/Sham control	54.1±2.04[a]	106.1±0.98[a]

I-R injury	7.3±1.96[b]	10.3±1.03[b]
d-Limonene 50	20.6±1.75[c]	28.1±1.16[c]
d-Limonene 100	33.3±1.5[c]	74.1±1.47[c]
d-Limonene 200	43.05±0.083[c]	91.3±1.21[c]
Edaravone	50.5±1.37[c]	102.8±6.52[c]

Values are expressed as mean ± S.E.M. [a]denotes $p<0.05$ compared to normal group, [b] denotes $p<0.05$ compared to surgical/sham control group, [c] denotes $p<0.05$ compared to I-R injury group (one way ANOVA followed by Tukey's test).

Effect of d-Limonene on brain TBARS, GSH, catalase, nitrite

Surgical/Sham control group animals showed higher brain TBARS and nitrite levels, whereas lower GSH and Catalase levels ($p<0.05$) as compared to the normal group. I-R injury animals showed higher brain TBARS and nitrite levels and lower GSH and Catalase levels ($p<0.05$) as compared to the surgical/sham control group, indicating the rise in oxidative stress due to successful induction of cerebral-ischemia reperfusion injury. The administration of d-Limonene (50, 100 and 200 mg/kg; *p.o.*) and edaravone for 30 successive days, prevented the rise in brain TBARS and nitrite levels and lower GSH and Catalase levels, in dose dependent manner and the results were statistically significant ($p<0.05$) as compared to I-R injury group, which indicates its anti-oxidant activity against generation of free radicals in cerebral ischemia-reperfusion injury in mice.

Table 3: Effect of d-Limonene on TBARS, GSH, Catalase and nitrite

Groups	TBARS (µMol/ml)	GSH (µMol/ml)	Catalase (Units[A])/mg Protein)	Nitrite (µMol/ml)
Normal	18±4.33	68.6±1.98	0.138±0.025	36.2±7.54
Surgical/Sham control	23.5±1.76[a]	55.8±1.47[a]	0.126±0.0046[a]	62.0±0.27 [a]
I-R injury	52.6±2.58[b]	12±1.09[b]	0.054±0.033[b]	121.7±7.33[b]
d-Limonene 50	44.3±1.21[c]	25.5±1.51[c]	0.090±0.0104[c]	82.6±2.22[c]
d-Limonene 100	36±4.77[c]	35.8±2.85[c]	0.102±0.0037[c]	66.3±4.58[c]
d-Limonene 200	26.5±2.07[c]	44.8±2.04[c]	0.111±0.006[c]	53.3±2.65[c]
Edaravone	21.3±3.26[c]	52.3±1.03[c]	0.120±0.004[c]	41.7 ±4.33[c]

Values are expressed as mean ± S.E.M. [a]denotes $p<0.05$ compared to normal group, [b] denotes $p<0.05$ compared to surgical/sham control group, [c] denotes $p<0.05$ compared to I-R injury group (one way ANOVA followed by Tukey's test).

Effect of d-Limonene on Cerebral Infarct area:-

Surgical/Sham control animals showed slightly increase in 3.21% cerebral infarction area in the brain as compared to the normal group. The significant increase in % infraction volume of I-R injury animals 32.01% ($p<0.05$) as compared to the normal group, due to occlusion of carotid

artery and further reperfusion which leads to decrease in blood flow to the brain increases the infarct area in the brain. The administration of d-Limonene (50, 100 and 200 mg/kg; *p.o.*) and edaravone significantly decreased the percentage of the infarct area to 23.29% ($p<0.05$), 15.34% ($p<0.05$), 9.64% ($p<0.05$) and 5.64% ($p<0.05$), respectively.

Table 4: Effect of d-Limonene on cerebral infarct size

Groups	% Infarction
Normal	0%
Surgical/Sham control	3.21%
I-R injury	32.01%[a]
d-Limonene 50	23.29%[b]
d-Limonene 100	15.34%[b]
d-Limonene 200	9.64%[b]
Edaravone	5.64%[b]

Values are expressed as mean ± S.E.M. [a] denotes $p<0.05$ compared to surgical/sham control group, [b] denotes $p<0.05$ compared to I-R injury group (one way ANOVA followed by Tukey's test).

DISCUSSION

In the present study, we evaluated the neuroprotective effect of administration of d-Limonene in mice against the development of cerebral ischemia-reperfusion injury in the mice brain. Passive avoidance behaviour based on negative reinforcement is used to examine long-term memory in animals[46]. Administration of d-Limonene to I-R injury mice for 30 days showed significant increase in the SDL which indicates the memory improving effect of d-Limonene. Furthermore, I-R injury mice exhibited anxiety in elevated zero maze test. EZM is a sensitive tool to study the anxiety related behavior[45], which is based on the principle that rodents are generally unwilling to enter in the open arms of the maze, considering their aversion to open spaces. Administration of d-Limonene to I-R injury mice for 30 days significantly showed anxiolytic actions in EZM in a dose dependent manner. In this study, I-R injury showed weakened muscle grip strength and less loco-motor activity on rotarod apparatus and actophotometer as shown by fewer falls off time and less scoring on actophotometer, which indicates loss of muscle co-ordination response of mice Administration of d-Limonene in different doses to I-R injury mice for 30 days significantly increased fall of time and loco-motor score in animals.

Cerebral ischemia-reperfusion injury in mice, showed higher oxidative stress in the present study, I-R injury showed increased level of TBARS, nitrite and decreased level of GSH, and Catalase. The biochemical evidences of the present study suggested that, d-Limonene may have exerted its neuroprotective action by acting as free radical scavenger, by reducing oxidative stress and by

interrupting with lipid peroxidation processes in the elevated levels of TBARS and nitrite levels and increase the catalase, and GSH levels in I-R injury mice. However, in ttc staining the tetrazolium salt is reduced by the enzymes into a red, lipid soluble formazan. Viable tissues therefore stains deep red while the ischemic zone is known to progress to a dense fibrous scar with no viable muscle fibres, remains unstained. A high percentage of mean infarct size was observed in I-R injury animals when compared to Surgical/Sham control group. Animals treated with d-Limonene 50 showed low infarct area with reduced staining when compared to I-R injury animals. Animals treated with d-Limonene 100 & 200, both showed low infract area when compared to I-R injury animals. Animal treated with Edaravone showed a moderately low infarct area with reduced staining, respectively when compared to I-R injury animals.

Oxidative stress is involved in the pathophysiology of stroke. During the past two decades, accumulating research into the complex mechanisms of ischemic stroke has indicated that excessive reactive oxygen species (ROS) production and subsequent oxidative stress play harmful roles during cerebral I-R injury[47, 48.] Several studies have shown that oxidative stress contributes to brain injury due to ischemia-reperfusion[49, 50, 51]. Reperfusion in the brain after ischemia also induces an inflammatory response that may exacerbate the initial levels of tissue damage. There are a number of possible mechanisms by which post-ischemic inflammation could cause damage, including the production of toxic mediators such as NO by activated inflammatory cells and vascular occlusion by neutrophils[52], free radical generation following ischemic insult and reperfusion can inhibit the Na^+ K^+ pumps, causes mitochondrial changes, damage proteins, lipids and nucleic acids, resulting in the inactivation of some enzyme activities, disruption of ion homeostasis and modification of genetic apparatus and apoptotic death[53]. Many therapeutic strategies that have successfully limited or prevented I-R injury in controlled, experimental models have yielded equivocal results in clinical practice or have not reached human clinical trials. Furthermore, few studies have examined the efficacy of combined strategies in attenuating I-R injury. Thus, at present, timely reperfusion of strategies to attenuate I-R injury such as ischemic preconditioning, antioxidants therapy, anti-complement therapy, antileukocyte therapy, anti-inflammatory agents, free radical scavengers, glutamate release inhibition, potassium channel activation, GABA agonism, serotonin agonism, opiate antagonism, membrane stabilization[54]. Various studies in the literature reported antioxidants[29], antidiabetic[30], anticancer[29], antifungal[31], anti inflammatory[32], anti stress[33], GERD[34], dissolve cholesterol containing gallstones[35] activity of d-Limonene.

CONCLUSION

In conclusion, these findings suggest that d-Limonene prevents ischemia-reperfusion injury induced neuronal damage by virtue of its neuroprotective and antioxidant properties.

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