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Protective role of hispolon pyrazole and hispolon monomethyl ether pyrazole in electromagnetic radiation-induced behavioral, neurochemical, oxidative, and histological changes in rats

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ABSTRACT

Introduction: Radiofrequency electromagnetic radiation (RF-EMR) from mobile phones was reported to cause neurological damage. Hispolon pyrazole (HP) and hispolon monomethyl ether pyrazole (HMEP) were tested for their RF-EMR protection in rats.
Methods: Juvenile Wistar albino rats were exposed to the mobile phone generating 2400 MHz radiation with a maximum power output of 2 W/kg (Specific absorption rate 1.6 W/kg) for 90 days at a rate of 2 hours/day, treated with HP and HMEP at 20 and 40 mg/kg body weight. The elevated plus maze (EMT) test was used for anxiety and exploration evaluation, the forced swim test (FST) for depression, the Morris water maze test and Y-maze test for learning and memory. The oxidative stress markers like glutathione, superoxide dismutase (SOD), catalase (CAT), and malonaldehyde (MDA), and the neurotransmitters such as gamma-aminobutyric acid, glutamate, dopamine, and acetylcholinesterase along with histopathology in the cortex, striatum, and hippocampus were evaluated to establish the mechanism of the neuronal alterations of HP and HMEP against RF-EMR-induced damage.
Results: In the current investigation, HP at a higher dose of 40 mg/kg and HMEP at both doses significantly reduced the oxidative stress generated by RF-EMR from mobile phones and altered neurobehavioral, neurotransmitter, and histological alterations.
Conclusion: Based on the findings, HP and HMEP at a dose of 40 mg/kg are protective agents against long-term, continuous mobile phone use and can be regarded viable therapeutic agents.

Implication for health policy/practice/research/medical education:
The current study demonstrated that hispolon pyrazole (HP) and hispolon monomethyl ether pyrazole (HMEP) have neuroprotective potential against RF-EMR from mobile phones. These chemicals might be used to develop neuroprotective drugs.
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Introduction

Over the past two decades, the world had and is witnessing the rapid evolution of telecommunication technology from the 2G Global System for Mobile (GSM) to the 4G Long-Term Evolution-Advanced (LTE-A) system (1). Excessive utilization of mobile phone as multipurpose device led to the development of advanced features that needs a higher generation of technology that can provide extended connectivity and speed at higher bandwidths. Many studies have reported the harmful effects of radiofrequency electromagnetic radiation (RF-EMR) from mobile phones on various organ systems like the brain (2-4), kidney (5,6), ear (7), heart (8), liver (9,10), and reproductive organs (6). Most mobile phones are used close to the head, and the harmful effects of the RF-EMR released on the brain are a significant concern. The RF-EMR at a frequency range of 900 to 2200 MHz can penetrate deeply into the brain, causing fatigue, headaches, and decreased cognitive and

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behavioral abilities (11-15). No drug was specifically used to treat the adverse effects caused by EMR. Yet, many researchers have been experimenting with new chemical entities of both natural and synthetic origin to determine whether the harmful effects of RF-EMR might be mitigated (10,16-20).

Hispolon, a polyphenolic compound isolated from medicinal mushrooms like *Phellinus igniarius, Phellinus linteus*, and *Inonotus hispidus* (21-23), has been reported to treat pain and inflammation (24,25), diabetes (26), oxidative stress (27), cancer (28), viral infections (29), and also proven to have hepatoprotective (27), immunomodulatory (30), and cerebroprotective (31) activities. Several novel compounds were created and tested based on the stated pharmacological properties for diverse activities. Due to the similarity of Hispolon to half curcumin, several studies have examined the effectiveness of hispolon and its different derivatives, such as hispolon pyrazole (HP), HME (hispolon monomethyl ether), and hispolon monomethyl ether pyrazole (HMEP), as powerful antioxidants and ROS scavengers in cell-free systems (32) and genotoxicity in irradiated cells, of them, HP and HMEP were found to be antioxidant (33). Radiation exposure raises intracellular ROS levels and damages DNA, which results in cell death (34). However, any substance that may lower ROS concentrations or change how ROS damages DNA can shield cells from EMR.

Thus, this study aimed to evaluate the protective role of HP and HMEP in behavioural, neurochemical, oxidative, and histological damages caused by mobile phone radiation.

Materials and Methods

Materials

Synthetic derivatives of hispolon (HP and HMEP) were provided by Natsol Laboratories Pvt Ltd, Vishakhapatnam, as gift samples. Thiobarbituric acid was prepared from Otto, India; glutathione, eosin, and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were procured from Loba Chemie, India. Pyrogallol was prepared from Sisco Research Laboratories Pvt Ltd, India, glutamate, GABA, and dopamine from HiMedia, India, haematoxylin from Merck Specialities Pvt Ltd, Mumbai, India, diethyl ether from Thermo Fisher Scientific India Pvt Ltd, India. Analytical-grade chemicals with the possible highest purity were used in this study.

Animals

Neonatal albino Wistar rats of both sexes were bred from the animals procured from Mahaveer Enterprises, Hyderabad, India. Until 21 days, the neonatal rats were housed along with the parent rats, after which the experimental rats were housed in polyacrylic cages with six animals per cage. They were provided with a standard pellet diet from Mahaveer Enterprises, Hyderabad, India, and water ad libitum. Standard conditions of temperature (25°C ± 5°C), relative humidity (55 ± 10%), and a 12:12 hours light-dark cycle were maintained throughout the study. Institutional Animal Ethical Committee approved the experimental protocol of Vignan Pharmacy College, Vadlamudi (Reg No. 1499/PO/Re/S/11/CPCSEA).

Experimental design

Experimental animals of both gender at an equal ratio were randomly assigned to 6 independent groups containing eight animals each. All the treatment interventions were blinded to the researcher until statistical analysis. After blinding, the group I received 1% tween 80 as vehicle control, group IV received distilled water as it served as sham control, and groups II and V received HP 20 and 40 mg/kg body weight, respectively. Groups III and VI received HMEP 40 and 20 mg/kg body weight, respectively, 30 minutes before the daily mobile phone radiation exposure. Sham control group animals were placed in similar exposure conditions while the mobile phone was switched off. Animals of all groups were habituated to exposure cages for one week before exposure to avoid procedure-related stress.

Exposure protocol

Except for the control and sham control groups, all groups were subjected to RF-EMR emitted by a mobile phone equipped with the Voice over Long-Term Evolution (VoLTE) - 4G communication technology, which emitted 2400 MHz radiation with a maximum power output of 2 W/kg (Specific absorption rate [SAR]; 1.6 W/kg). The cell phone was positioned 2 cm away from the animals on the upper side of the grill mesh of a small polycarbonate cage. This was deliberately done to prevent rodents from getting close to the phone. Four animals that could walk about freely were housed in each exposure cage. Rats were exposed to radiation emitted by the mobile phone in the talking mode, receiving calls from another phone (silent mode) continuously for two hours per day for 90 days (35) between 09:00 to 11:00 AM daily (12). To prevent exposure to mobile phone radiation, animals in the sham control group were given the same conditions of mobile phone exposure, switched to standby mode, and kept in a separate room. There were no other electronic devices, such as a computer, notebook, camera, or RF-EMR generating sources, in the RF-EMR exposure room.

Experimental procedure

Animals were subjected to treatment and radiation exposure simultaneously. After 90 days, each animal was observed for change in body weight and behavior assessments such as anxiety using elevated plus maze (EPM), depression by forced swim test (FST), locomotion and memory by Y maze, and Morris Water Maze (MWM), respectively. To prevent the influence of prior testing on the results, all of the animals' behavioral measures were

evaluated between 9:00 AM and 5:00 PM with an hour between tests. Following the behavioral evaluations, all the animals were anesthetized using a high dose of pentobarbital. The brains were then isolated and tested for oxidative stress, neurochemicals (acetylcholinesterase, GABA, glutamate, and dopamine), and histopathology in the cortex, striatum, and hippocampus of rats.

Gravimetry

Determining the impact of mobile phone radiation on animal body weight is made possible by measuring body weight (BW). A precision weighing balance from Essae-Teraoka Pvt. Ltd.'s PG/FB series was used to measure the animal's body weight before exposure to mobile phone radiation and after every 30 days until the end of the experiment. The % change in the body weight was measured using the following equation (36).

$$\%\text{BW change}=\frac{(\text{Initial BW-Present BW})}{\text{Initial BW}}\times 100$$

where BW stands for bodyweight.

Behavioral assessments

Assessment of anxiety behavior using EPM test

The fear and anxiety-like behaviors were assessed using the EPM test based on the natural preferences for open and elevated areas (37,38). A standard EPM for rats was used with dimensions of 50 cm ×10 cm × 40 cm (l×b×h) for closed arms and 50 cm × 10 cm (l×b) for open arms with a 10 cm × 10 cm common central platform, standing 50 cm above the ground and made of wood. All the observations were recorded in a sound-free room illuminated with light. Animals were familiarised with the observation room and the test apparatus half an hour before the observations. Rats were introduced to the central platform of the maze facing the open arm and allowed to explore the maze for 5 minutes. After that, the rat was returned to its cage. The maze was cleaned with 20% alcohol to avoid a putrid odor index before testing the new animal. The rat's movements were captured in later analyses using a digital camera. The number of open and closed-arm admissions and the length of time spent in the open arms were indicators of fear and anxiety-like behavior. When a rat reached an arm with all four legs, it was counted one entry in that arm (2). Reduced anxiety was suggested by the increased number of entries and the length of their stays in the open arms (37). The percent of time spent in either of the arms was calculated using the following formula:

$$\%Time\ spent=\frac{Time\ spent\ in\ each\ arm\,(s)}{Total\ observation\ time\,(s)}\times 100$$

Assessment of depressive behavior using FST

The rats were each placed in a cylinder that was 45 cm tall, 20 cm wide, and contained 25 cm of water that was kept at a constant temperature of 25 ± 2°C. Two swimming sessions were held: a 15-minute pre-test and a 5-minute test 24 hours later. After 5 minutes, the animals were removed from the water cylinder, and the total time the animals were immobile was recorded. A rat was observed passively floating in the water and deemed immobile (39-41).

Assessment of exploratory, locomotor, and spatial working memory using Y-Maze

The Y-maze has been used to test spatial working memory in rodents (42). The three identical wooden arms that made up the Y-maze had the measurements of 30 cm in length, 8 cm in width, and 15 cm in height, with a 120° angle between them. The arms were given at random the start arm (S), novel arm (N), and familiar arm (F). In the first trial, the novel arm was closed, and the animals were free to explore the start and known arms for 15 minutes. In the second trial, the rats were given an uninterrupted 5 minutes to explore all three arms after an inter trail interval (ITI) of 1 hour; the number and sequence of arm entrances and the length of time spent in the novel arm for a total of 5 minutes were recorded. Between experiments, water spray was used to clean the maze arms and eliminate any lingering smells. The total number of arm entries showed locomotor activity; exploratory activity was indicated by the length of time spent in the novel arm, and the sequential entries indicated spontaneous alteration behavior into the three arms on overlapping triplet sets (SFN, FNS, and NFS, etc.). The following formula was used to calculate the working memory and cognitive behavior (43-45):

$$\%Alterations=\frac{Number\ of\ \ positive\ alterations\ made}{Total\ number\ of\ arm\ entries-2}\times 100$$

Assessment of memory and learning in animals using M W M

The MWM test was used to assess long-term spatial learning and memory in rodents (46,47). The maze was a wide-open circular plastic tank with a 150 cm diameter and 62.5 cm height, filled with opaque water of 23±1°C to 40 cm. The maze was split into four equal quadrants by two fictitious perpendicular lines using the Voorhees and Williams approach (East (E), West (W), North (N), and South (S)). The location of the platform (11 cm in diameter) was selected in the southwest quadrant, 1.5 cm below the water's surface (48). This test was divided into two sessions (acquisition phase and probing test) on consecutive days. The acquisition phase lasted five days and consisted of four trials per day. Each rat has 60 seconds time to find out the submerged platform and 30 seconds to stay there. If the rat did not find the hidden platform within 60 seconds, it was guided to it and permitted to remain there for 30 seconds. The procedure was repeated at each of the four starting points. The rats were released into the maze facing the pool's wall to begin

the trial. The latency to locate the escape platform was limited to one minute. The latency to find the platform during the acquisition phase was reported daily. During the probing test, the hidden platform was taken out of the water, allowing the rat to swim for 90 seconds. The average distance to the previous platform, the latency, the frequency, and the time spent in the target quadrant were all recorded.

Preparation of brain homogenates

After completing the experimental protocol, all the animals were deeply anesthetized with diethyl ether and transcardially perfused with ice-cold phosphate-buffered saline. This was followed by decapitation to isolate brains from the rats. Isolated brains were separated into two hemispheres, and the striata, cortex, and hippocampus were separated. The sections were then homogenized with ice-cold phosphate-buffered saline for biochemical and enzymatic estimations. The remaining half was treated concurrently using the abovementioned methods and homogenized with 80% ice-cold ethanol for neurochemical assessments (40,49).

Assessment of biochemical markers

For biochemical examination, the striatum, cortex, and hippocampus of sectioned brain hemispheres were individually homogenised in 10% w/v phosphate buffer (pH 7.4). Following centrifugation at 3354 × g, homogenates were divided into aliquots using the technique outlined by Ohkawa et al (50), reduced glutathione (GSH) by Ellman (51), which was measured at absorbance 412 nm, superoxide dismutase (SOD) assay described by Marklund and Marklund (52), catalase (CAT) activity measured at 240 nm (53), an indirect estimate of acetylcholinesterase (AChE) activity; release of thiocholine was measured by Ellman et al (54) method.

Assessment of neurochemicals

As previously mentioned, the levels of glutamate and GABA in the cortex, striatum, and hippocampus were divided and sectioned into the brain hemispheres. Briefly, all three of the brain samples of three areas were rinsed with ice-cold 80% ethanol before being homogenized in the same solution to form 10% w/v homogenates for each. Centrifuging homogenates at 8586 g, the separated supernatants and sediments were recovered. GABA and glutamate were extracted from sediments using several extractions with 80% ice-cold ethanol. All of the samples' ethanol was completely evaporated using 3 ml of the abovementioned extract, and the leftover was then reconstituted with 100 ml of distilled water.

Along with test samples, standard concentrations (2 mM) of glutamate and GABA were prepared and spotted by paper chromatography. The chromatography papers that had been stained were placed in a chamber that had been saturated with a mixture of butanol, acetic acid, and water (12: 3: 5 v/v) as the solvent. Chromatography papers were dried after development, and the procedure was repeated. The ninhydrin reagent was sprayed onto the dried papers, which were then dried for four minutes at 100°C. The sample spots were matched to the standards (glutamate and GABA), and the spots were cut out to extract the sample's contents with 0.005 % $CuSO_4$ in 75 % ethanol. Using a spectrophotometer, the elute's absorbance was measured at 515 nm (55,56).

Dopamine (DA) was estimated using the method described by Schlumpf et al (57). Wet tissue was weighed, homogenized for 1 minute in 5 mL of HCl-butanol, and then centrifuged at 2000 RPM for 10 minutes. One millilitre of the supernatant phase was taken as an aliquot and put in a centrifuge tube with 2.5 mL of heptane, and 0.3 mL of 0.1 M HCl. The tube was centrifuged under the same conditions as above after 10 minutes of vigorous shaking to separate the two phases, and the extra organic phase was discarded. The DA assay required 0.2 mL of the aqueous phase. The above procedures were completed using ice cubes in a 0 to 8°C environment. 0.05 mL of 0.4 M HCl, 0.1 mL of EDTA/sodium acetate buffer (pH 6.9), and 0.1 mL of iodine solution (0.1 M in ethanol) for oxidation were added to the 0.2 mL of the aqueous phase. After two minutes, the reaction was stopped by adding 0.1 mL of Na_2SO_3 solution. 0.1 ml of acetic acid was added after 15 minutes. The solution was then heated to 100°C for 6 minutes after the sample reached room temperature, and the spectrofluorometer was then used to read the excitation and emission spectra between 330 and 375 nm (55).

Histopathological studies

The fully intact brain was placed in formalin (10% v/v). Three-mm thick blocks of the striatum, cortex, and hippocampus tissues were cut, and the blocks were then set in paraffin. Hematoxylin and eosin (H&E) were used to produce and stain the brain slices, which ranged in thickness from 5 to 10 microns. It was done in accordance with the established protocol to accomplish hematoxylin and eosin staining. The histology was examined under a digital microscope after staining, and pictures were taken (55).

Statistical analysis

All the data were subjected to a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using SPSS Version 26 software; a $P < 0.05$ was considered statistically significant. Graphing was done using Origin V9 software. The analyzed data were represented as mean ± standard error of the mean (SEM).

Results

Effect of hispolon compounds on body weight of rats exposed to RF-EMR

In the present study, animal body weights were observed

throughout the experiment and recorded on the 0th, 30th, 60th, and 90th days. The mean percent increase in the animal body weight was measured for each group and presented in Figure 1. Animal body weight was significantly lower in the radiation control group than in the normal control group, proving that mobile phone radiation had an effect on it. As opposed to the radiation control group, the treatment groups HP 20 and HMEP 40 demonstrated a substantial increase in body weight ($P<0.01$ and $P<0.001$, respectively). In the other treatment groups (HP 40 and HMEP 20), there was an insignificant increase in body weight compared to the radiation control.

Behavioural analysis

Effect of hispolon compounds on the anxiety (EPM test) of rats exposed to RF-EMR

Animal entries substantially increased after exposure to radiation from mobile phones. When compared to the normal control group, they spent more time on the closed arm of EPM while decreasing the entries and time spent on the open arm ($P<0.001$) (Figure 2A & 2B). Compared to the radiation control group, the treatment with HP and HMEP at dosages of 20 mg and 40 mg dramatically reversed ($P<0.001$) the effect of mobile phone radiation by boosting the decrease in animals' anxiety.

Effect of hispolon compounds on the depression (FST) of rats exposed to RF-EMR

When compared to the healthy control group, animals exposed to mobile phone radiation had significantly reduced hind limb movement and were immobilized for a longer period of time ($P<0.001$) (Figure 3). Compared to the radiation control group, treatment with HP and HMEP at low and high doses significantly reduced the animals' immobility ($P<0.01$ and $P<0.001$).

Effect of hispolon compounds on the memory of the rats exposed to RF-EMR

Y Maze test

Short-term spatial memory was affected by the mobile phone radiation by significantly reducing ($P<0.001$) the % spontaneous alternations in the rats compared to

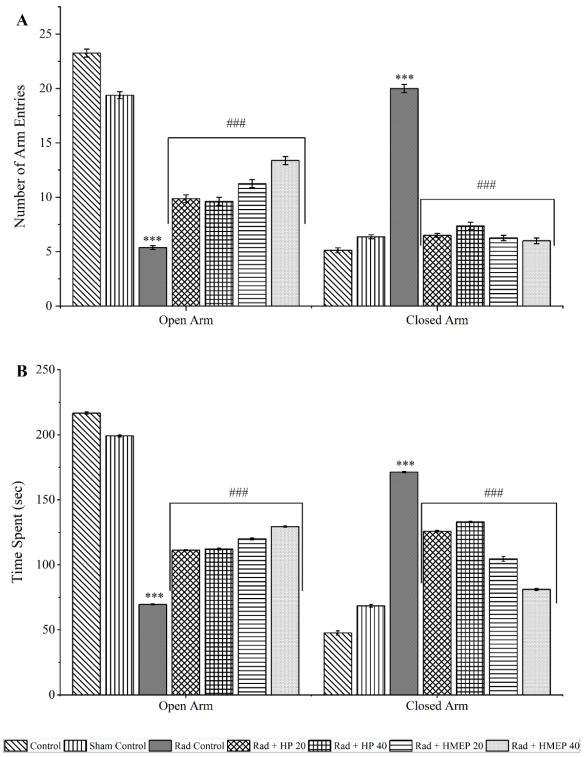

Figure 2. Effect of hispolon compounds on the anxiety of rats exposed to RF-EMR. (A) Number of arm entries and (B) Time spent in open and closed arms of EPM. Values are reported as mean ± SEM (n=8). *** $P<0.001$ when compared to the normal control, ### $P<0.001$ when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

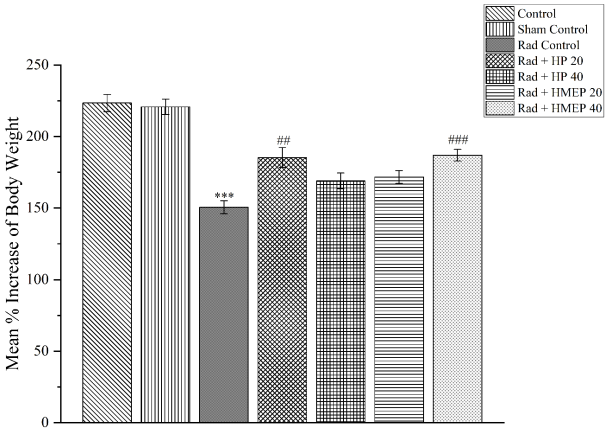

Figure 1. Effect of hispolon compounds on the body weight of rats exposed to RF-EMR. Values are reported as mean ± SEM (n=8). *** $P<0.001$ when compared to the normal control, ## $P<0.01$ and ### $P<0.001$ when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

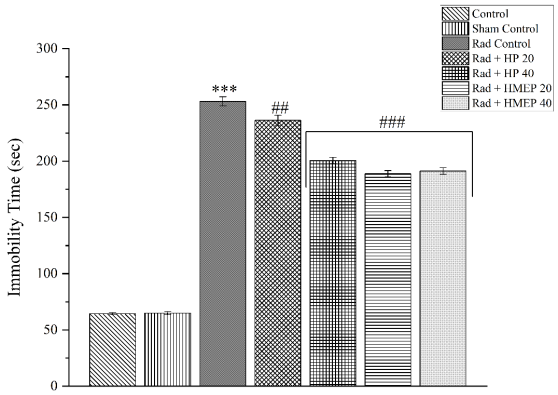

Figure 3. Effect of hispolon compounds on depression (FST) of rats exposed to RF-EMR. Values are reported as mean ± SEM (n=8). *** $P<0.001$ when compared to the normal control, ### $P<0.001$ when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

the normal control group. However, the treatment with HP and HMEP at the doses of 20 and 40 mg/kg (HP 20, HMEP 20, and HMEP 40) showed a significant increase ($P<0.001$) in % spontaneous alternations when compared to the radiation control group, indicating the improvement of the spatial working memory (Figure 4A). Similarly, the animals in the radiation control group showed a significant decrease ($P<0.001$) in exploring the novel arm compared to the normal control animals. This indicated decreased spatial reference memory by less exploring the novel arm. The treatment with HP and HMEP at doses of 20 and 40 mg/kg significantly increased ($P<0.001$) the percent entries into the novel arm signifying the improvement of the spatial reference memory when compared to the radiation control animals (Figure 4B). It was also observed that the animals were continuously moving in the arms, indicating increased locomotor activity in the radiation control group, which may be accounted for hyperactivity in animals. In contrast, HMEP 40 significantly ($P<0.01$) decreased the total arm entries (Figure 4C) by increasing the time spent. Furthermore, the percent time spent in the novel arm decreased significantly ($P<0.001$) in the radiation control group compared to the normal control animals. The decrease in short-term memory was improved significantly in all the treatment groups by increasing the percent time spent in the novel arm in the Y maze (Figure 4D).

MWM test

The measurement of escape latency before locating the hidden platform for over five days showed an improvement in acquiring spatial information, as shown in Figure 5A. Radiation exposure to the animals showed a significant increase in the escape latency in the radiation control group compared to the normal control group ($P<0.001$). Similarly, the animals exposed to radiation alone also increased the latency to the target quadrant ($P<0.001$) (Figure 5B) and decreased the time spent and frequency of entries into the target quadrant, indicating the impairment of spatial acquisition and retrieval of memory after a 5-day trial when compared to the normal group of animals (Figure 5C-5D). The treatment with HP and HMEP at all doses significantly decreased escape latency, while HMEP at all doses significantly improved ($P<0.001$) spatial learning of the animals compared to the radiation control.

Biochemical analysis

Effect of hispolon compounds on oxidative biomarkers in rats exposed to RF-EMR

Enzymatic antioxidant defence is represented by SOD and CAT; GSH represents non-enzymatic antioxidant defence; lipid peroxidation in the brain is represented by malondialdehyde (MDA). When compared to the healthy control group, the exposure of rats to mobile phone radiation for 2 hours continuously over a period of 90 days resulted in significant reductions ($P<0.001$) in the levels of GSH, SOD, and CAT and significant increased ($P<0.001$) the levels of MDA in the cortex, hippocampus, and striatum, both of which indicated oxidative stress in the brain. Treatment with HP at a dose of 20 mg/kg could recover the levels of GSH ($P<0.001$ and $P<0.05$) and MDA ($P<0.01$ and $P<0.01$) in the cortex and hippocampus, respectively, while GSH ($P<0.05$) alone in the striatum.

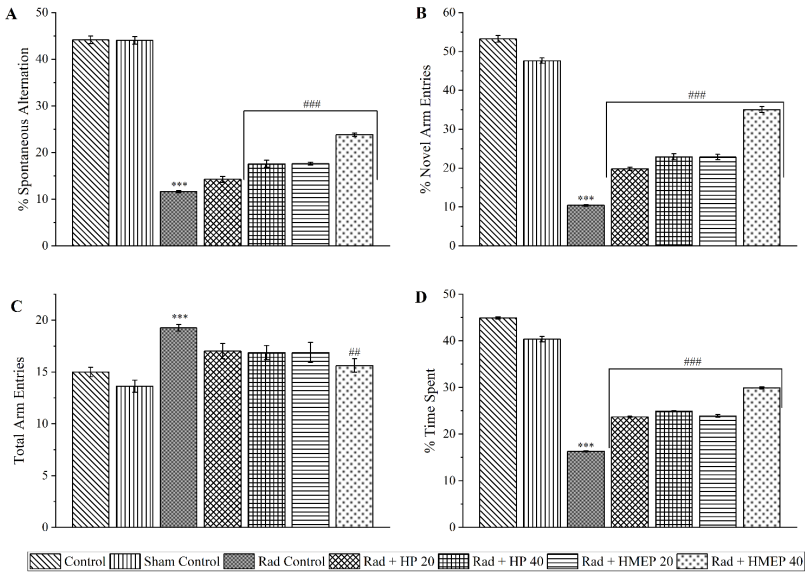

Figure 4. Effect of hispolon compounds on Y maze test of rats exposed to RF-EMR. A) % Spontaneous alternations, (B) % Novel arm entries, (C) Total arm entries, (D) % Time spent in the novel arm. Values are reported as mean ± SEM (n=8). *** $P<0.001$ when compared to the normal control, ## $P<0.01$ and ### $P<0.001$ when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

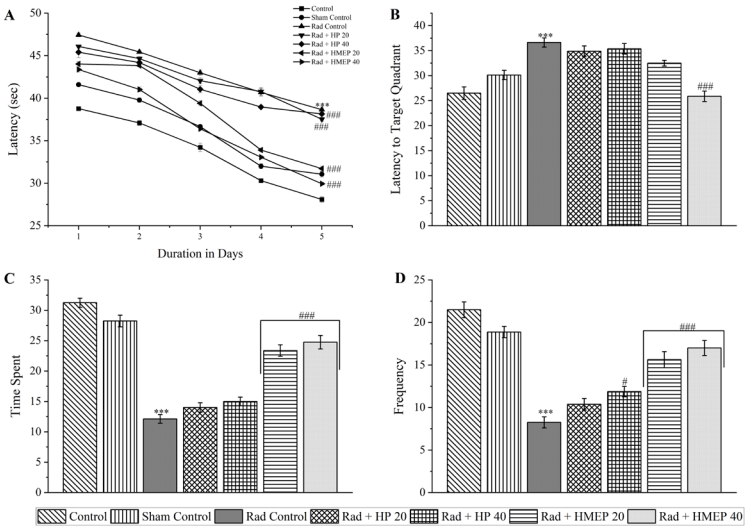

Figure 5. Effect of hispolon compounds on MWM test of rats exposed to RE-EMR. (A) Escape latency, (B) Latency to target quadrant, (C) Time spent, and (D) Frequency to target quadrant. Values were reported as mean ± SEM (n=8). *** $P<0.001$ when compared to the normal control, $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

However, at 40 mg/kg significantly recovered the levels of GSH ($P<0.001$ and $P<0.01$) and MDA ($P<0.001$) in the cortex, hippocampus, and striatum, respectively, when compared with the radiation control group. The treatment with HMEP 20 mg and 40 mg significantly recovered the GSH, CAT, and MDA ($P<0.001$) in the cortex and hippocampus. The data are presented in Figures 6, 7, and 8 for rat brains' cortex, hippocampus, and striatum.

Enzymatic estimation

Effect of hispolon compounds on AChE activity in the cortex, hippocampus, and striatum of the rats exposed to RF-EMR

The AChE activities in the animals' cortex, hippocampus, and striatum brains were estimated to assess the neuronal activity responsible for memory. AChE activity was significantly increased in the cortex, hippocampus, and striatum ($P<0.001$) relative to their respective controls in mobile phone radiation-induced animals (Figure 9). On the other hand, treatment with hispolon compounds, viz., HP and HMEP, decreased the AChE activity in a dose-dependent manner compared to the control animals. There was a significant decrease in the activity of AChE in the striatum and cortex regions of the brain when treated with HP and HMEP at a dose of 20 and 40 mg/kg ($P<0.001$), as well as HP 40 ($P<0.05$) and HMEP 20 and 40 at $P<0.001$ in the hippocampus. Comparatively, HMEP

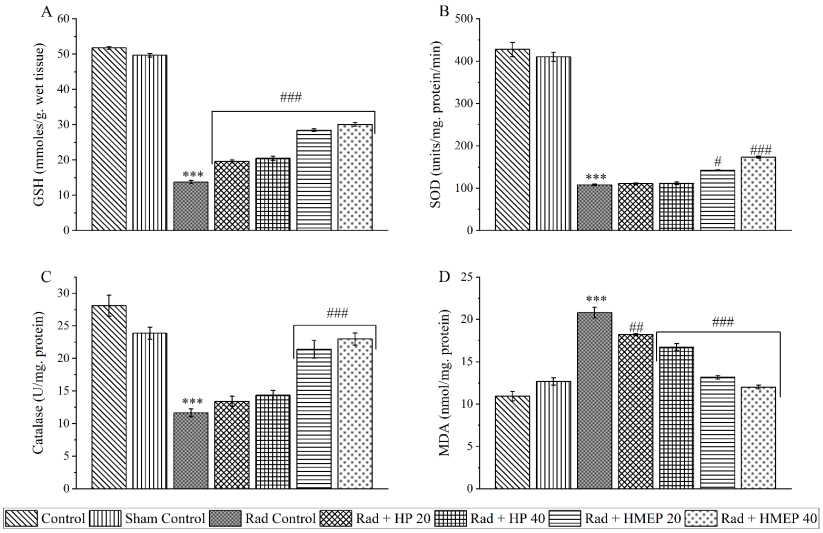

Figure 6. Effect of hispolon compounds on oxidative parameters in the cortex region of the rats exposed to RF-EMR. (A) GSH, (B) SOD, (C) Catalase, and D) MDA. Values are reported as mean ± SEM (n=8). *** $P<0.001$ when compared to the normal control, $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

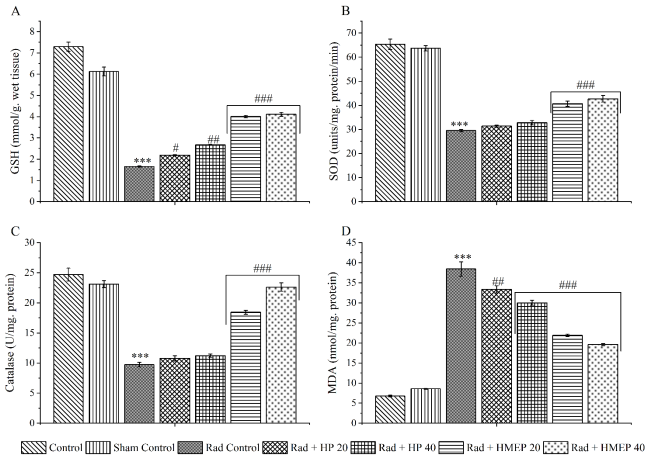

Figure 7. Effect of hispolon compounds on oxidative parameters in the hippocampus region of rats exposed to RF-EMR. (A) GSH, B) SOD, (C) Catalase, and (D) MDA. Values are reported as mean ± SEM (n=8). *** $P<0.001$ when compared to the normal control, $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

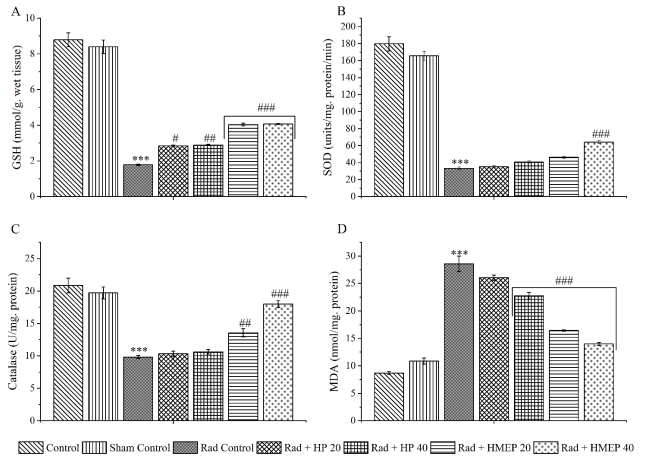

Figure 8. Effect of hispolon compounds on oxidative parameters in striatum region of rats exposed to RF-EMR. (A) GSH, (B) SOD, (C) Catalase, and (D) MDA. Values are reported as mean ± SEM (n=8). *** $P<0.001$ when compared to the normal control, $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

40 mg/kg treated group effectively lowered the activity of AChE in the cortex, hippocampus, and striatum.

Neurochemical analysis

Effect of hispolon compounds on GABA, glutamate, and DA levels in the cortex, hippocampus, and striatum of the rats exposed to RF-EMR

The results showed a significant decrease ($P<0.001$) in the levels of neurotransmitters like GABA and glutamate in the cortex, hippocampus, and striatal regions of the rat brains exposed to mobile phone radiation compared to unexposed brains (Figure 10). Furthermore, the treatment with HP and HMEP at two different doses (20 and 40 mg/kg), HP 40 in the cortex ($P<0.05$) and HMEP at both doses significantly increased the levels of GABA in the cortex ($P<0.001$), striatum ($P<0.01$ and $P<0001$), and hippocampus ($P<0.001$) respectively, when compared to the radiation control group (Figure 10A). HP significantly increased the levels of glutamate at low doses in the cortex ($P<0.05$) and high doses in the cortex and striatum ($P<0.001$ and $P<0.01$, respectively). HMEP at both doses significantly increased ($P<0.001$) the levels of glutamate in the cortex, striatum, and hippocampal regions of the brain when compared to the radiation control (Figure 10B). The DA levels were significantly increased ($P<0.001$) in the cortex and striatum while decreased in the hippocampal region in the brains of rats exposed to mobile phone radiation relative to the normal control. The treatment with HP (low dose) in the cortex ($P<0.01$), striatum ($P<0.001$), high dose in the cortex ($P<0.001$) and striatum ($P<0.001$), significantly lowered the DA levels, while increased in hippocampal ($P<0.05$) regions of brains when compared to the radiation control animals. On the other hand, HMEP at both doses significantly

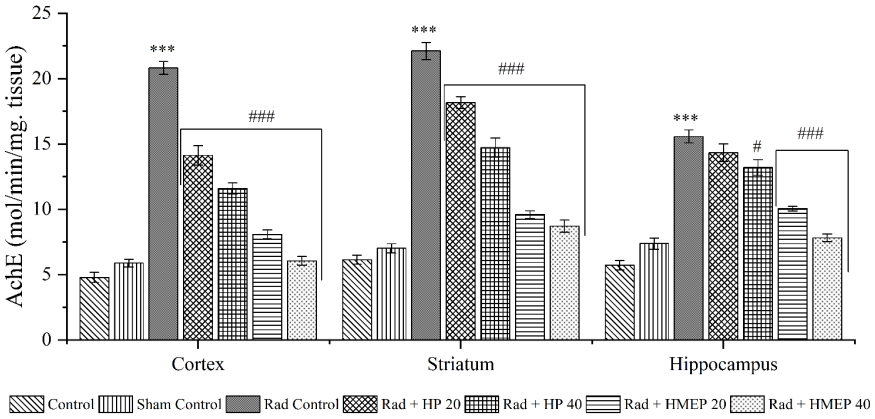

Figure 9. Effect of hispolon compounds on AChE activity in rats exposed to RF-EMR. Values are reported as mean ± SEM (n=8). ***P<0.001 when compared to the normal control, #P<0.05 and ###P<0.001 when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

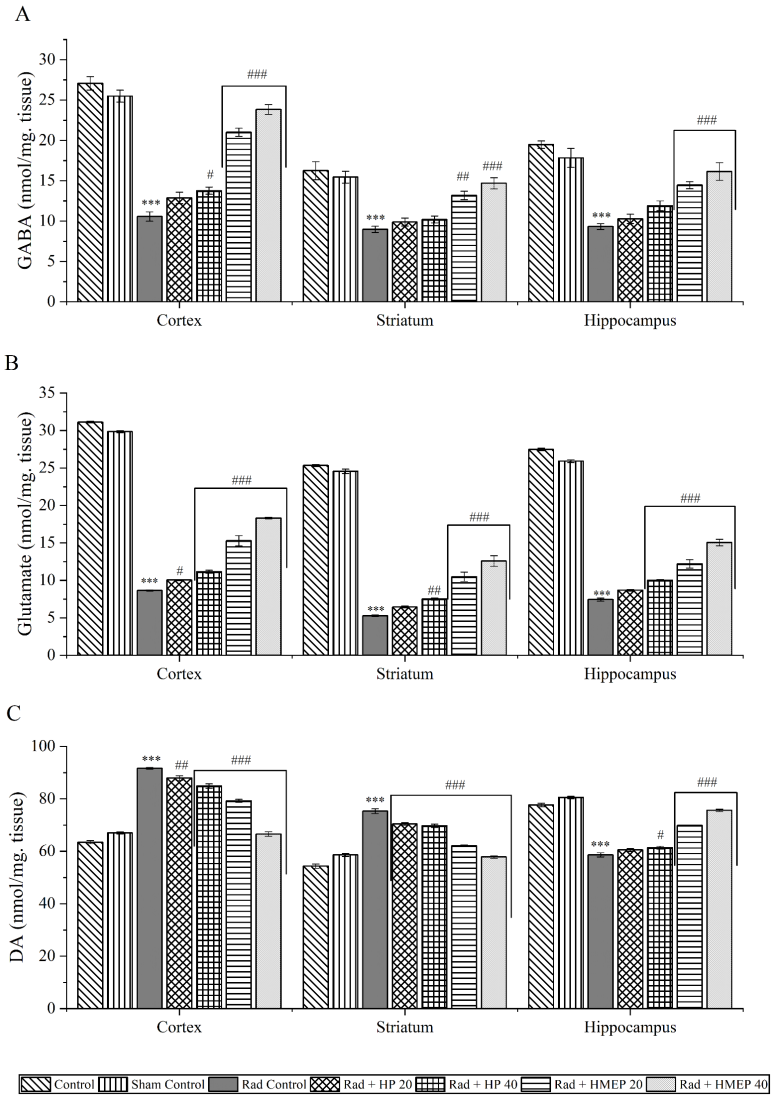

Figure 10. Effect of hispolon compounds on neurochemical levels in cortex, hippocampus, and striatum of the rats exposed to RF-EMR. (A) GABA; (B) Glutamate; (C) DA. Values were reported as mean ± SEM (n=8). *** P<0.001 when compared to the normal control, #P<0.05 and ###P<0.001 when compared to the radiation control group. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

lowered the DA in both the cortex and striatum ($P < 0.001$) while significantly increased in the hippocampal region of the brain when compared to the radiation control group (Figure 10C).

Histopathology

Effect of hispolon compounds on histological changes in the cortex and striatum of the rats exposed to RF-EMR

Examination of H&E-stained cerebral cortex and striatal sections of brains of normal control and sham control group animals (Figures 11 and 12 - Panels A&B) showed healthy histoarchitecture with single or double open-faced, rounded large centrally located nucleus and prominent nucleoli. On the other hand, the sections of the cortex and striatum of the brains exposed to mobile phone radiation, 2 hours daily for 90 days showed less viable cells, increased apoptotic cells, darkly stained pyramidal cells along with condensed cytoplasm with pyknotic nuclei, and vacuolization (Figures 11 and 12 - Panel C). Treatment with HP and HMEP at lower dose (20 mg/kg) showed neurofibrillary tangles, infiltration of inflammatory cells, and condensed cytoplasm with pyknotic nuclei and neurons with shorter dendrites. In comparison, at higher doses of HP and HMEP, restoration of histoarchitecture was observed with a smaller number of neuronal abnormalities in the cortical and striatal regions (Figures 11 and 12 - Panels D-G).

Effect of hispolon compounds on morphological changes in the hippocampus of the rats exposed to RF-EMR

The control and sham control group animals showed normal and healthy hippocampal pyramidal cells in the layers of *Cornu ammonis* (CA), CA1, CA2, CA3, and DG regions of the rats. Marked neuronal degeneration was observed in the CA areas and DG of the rat brains exposed to the mobile phone radiation characterized by vacuolization, condensed cytoplasm, darkly stained and pyknotic nuclei, and neurofibrillary tangles (Figure 13A). The treatment with HP and HMEP at higher doses markedly decreased neuronal degeneration compared to the radiation control group (Figure 13B and 13C).

Discussion

The present study demonstrated the neuroprotective potential of HP and HMEP on neurotoxicity induced by RF-EMR emitted from 2400 MHz mobile phone upon exposure for 90 days at a rate of 2 h/day. Studies have shown that mobile phone RF-EMR reproduces various behavioral, biochemical, and histopathological alterations (12,35). In our study, exposure to mobile phone radiation produced anxiety. It decreased exploratory activity in animals in the EPM test, where the animals avoided the open arm in terms of arm entries and time spent. In EPM, open-arm avoidance is the index of anxiety-like behavior (38). The exposed animals showed decreased the arm entries and time spent in open arms of an EPM and are par with the previous reports (2). On the other hand, the exposed rats exhibited immobility within a short time after being anxious, and the time of immobility was considerably increased, which can be observed in FST, indicating depressive behavior. Treatment with HP and HMEP at a low dose (20 mg/kg) and high dose (40 mg/kg) significantly increased the open arm entries and time spent in the EPM test and decreased the immobility time in the FST paradigm signifies the protective effect in behavioral changes caused by mobile phone radiation.

Additionally, MWM was used to evaluate the exposed rats' cognition and memory. In the trial sessions, the

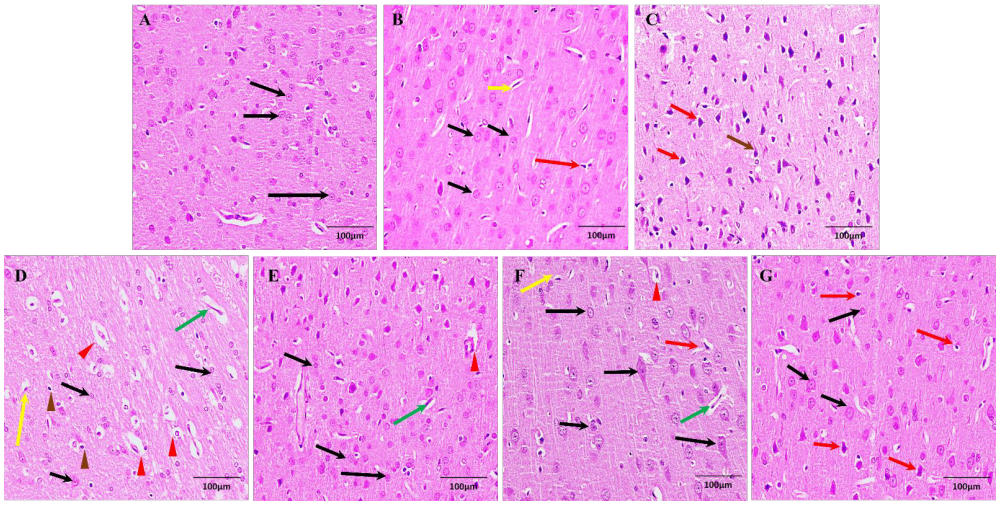

Figure 11. Effect of hispolon compounds on histological changes in the cortex of the rats exposed to RF-EMR. (A) Control, (B) Sham control, (C) Radiation control, (D) Rad + HP 20, (E) Rad + HP 40, (F) Rad + HMEP 20, (G) Rad +HMEP 40. Black arrow: Normal neuronal cells, red arrow: necrotic neurons, brown arrow: darkly stained pyramidal cells, yellow arrow: pyknotic, red arrowhead: vacuolization, brown arrowhead: inflammatory cells, green arrow: neurofibrillary tangles. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

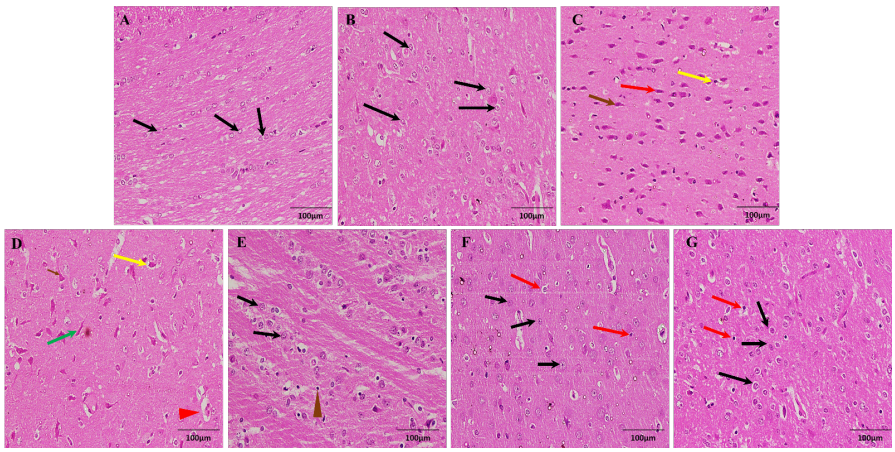

Figure 12. Effect of hispolon compounds on histological changes in striatum of the rats exposed to RF-EMR. (A) Control, (B) Sham Control, (C) Radiation Control, (D) Rad + HP 20, (E) Rad + HP 40, (F) Rad + HMEP 20, (G) Rad +HMEP 40. Black arrow: Normal neuronal cells, red arrow: necrotic neurons, brown arrow: darkly stained pyramidal cells, yellow arrow: pyknotic, red arrowhead: vacuolization, brown arrowhead: inflammatory cells, green arrow: neurofibrillary tangles. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

exposed animals took longer than the control animals to reach the hidden platform. They spent less time in the target quadrant throughout the retention test; their latency time to get there was also longer. Despite five training sessions, the phone-exposed animals could not recall the hidden platform's precise location on the memory retention day. This illustrates the animals exposed to phones' poor spatial navigation skills and object-place combinations. In the HMEP therapy groups, this was observed to be reversed indicating an improvement in cognition and memory (58).

Further, the animals were subjected to a Y-maze test to assess spatial working memory. Mobile phone radiation affected the spatial reference memory and novel preference behavior by decreasing the percent spontaneous alternations, novel arm entries, and time spent, respectively. Despite the above effect, mobile phone radiation increased the total arm entries, which signifies the development of hyperactivity-like behavior, per the previous report (59). HP and HMEP treatment groups improved spatial working memory in this study.

The neural circuit serves as the structural basis of brain activity, and the brain works due to the interaction between several neurotransmitters and various brain regions. Neurotransmission, differentiation, and the establishment of neuronal circuitry are all aspects of brain development in which neurotransmitters play a role. As a result, RF-modulatory EMR's effect on the amounts of neurotransmitters in different brain regions may be extremely important for brain function. Numerous studies have shown that exposure to RF-EMR might lead to an imbalance of amino acid neurotransmitters in different brain areas (60,61). The results suggest that exposure to mobile phone radiation for longer alters the neurochemicals in rats' cortex, hippocampus, and brain striatum. Our study is also more or less similar to other studies, which invariably altered major neurotransmitters.

In the present study, for instance, RF-EMR reduced the GABA in the cortex, striatum, and hippocampus, which controls various bodily activities, including regulating memory, emotion, and sleep, as well as hypertension, fatigue, and algesia (62,63). Radiation may interfere with GABA's ability to regulate the central nervous system, leading to an imbalance between excitement and inhibition. Our findings are consistent with earlier studies in that exposed rats had lower GABA levels, indicating inhibitory activity has been impaired, leading to anxiety (61,64).

Similarly, radiation decreased a major excitatory neurotransmitter, glutamate levels in the cortex, striatum, and hippocampus, which is responsible for learning and memory. It also forms GABA and antioxidant glutathione (65). Similar to the previous studies (66), exposure to mobile phone radiation in the current study decreased glutamine levels in the cortex, hippocampus, and striatum, and was also linked to the decrease in the levels of GABA. The current study suggests that RF-EMR might decrease the excitatory amino acid neurotransmitters in the hippocampus, altering the excitatory-inhibitory balance of neurons and damaging learning and memory.

Furthermore, the hypothalamus and pituitary gland also contain DA, a crucial neurotransmitter. It is primarily responsible for activities related to reward, learning, emotion, motor control, and executive functions in the brain (67). According to this study, RF-EMR exposure can cause the hippocampus to produce less dopamine, which could lead to a decline in learning and memory. Following exposure to EMR, elevated levels in the cortex and striatum may be a factor in developing schizophrenic-like behavior. Higher doses of HP and HMEP may lessen the behavioral abnormalities that result from exposure to mobile phone radiation in exposed rats' brain areas.

The AChE enzyme activity was also measured as part of this protocol, which is an indirect way to examine the role of acetylcholine in cognition and memory. AChE is essential for cholinergic neurotransmission because it hydrolyzes acetylcholine to stop the transmission of nerve impulses (68). These changed neurotransmitters also impacted the study's functional status (neurobehavioral). Additionally, it is possible to hypothesize that the changed cholinergic activity in the hippocampus serves as the neurological underpinning of behavioral changes. In the current investigation, the exposed brains' cortex, striatum, and hippocampi showed enhanced cholinesterase activity. A detrimental impact of RF-EMR exposure may affect the regulation and maintenance of learning and memory as the hippocampus processes storing and keeping information during the learning pathway (69). Additionally, a recent study showed that depression was one of the neurological conditions caused by RF-EMR exposure (70). From the above results, it can be concluded that HMEP at both doses could decrease the AchE activity in the regions of the brain.

The pathophysiology linked to RF-EMR exposure in brain cells has a crucial impact on the etiology and progression of oxidative damage (19,71). GSH depletion was seen after exposure to RF-EMR. The current study demonstrates the oxidizing conditions of the brain by

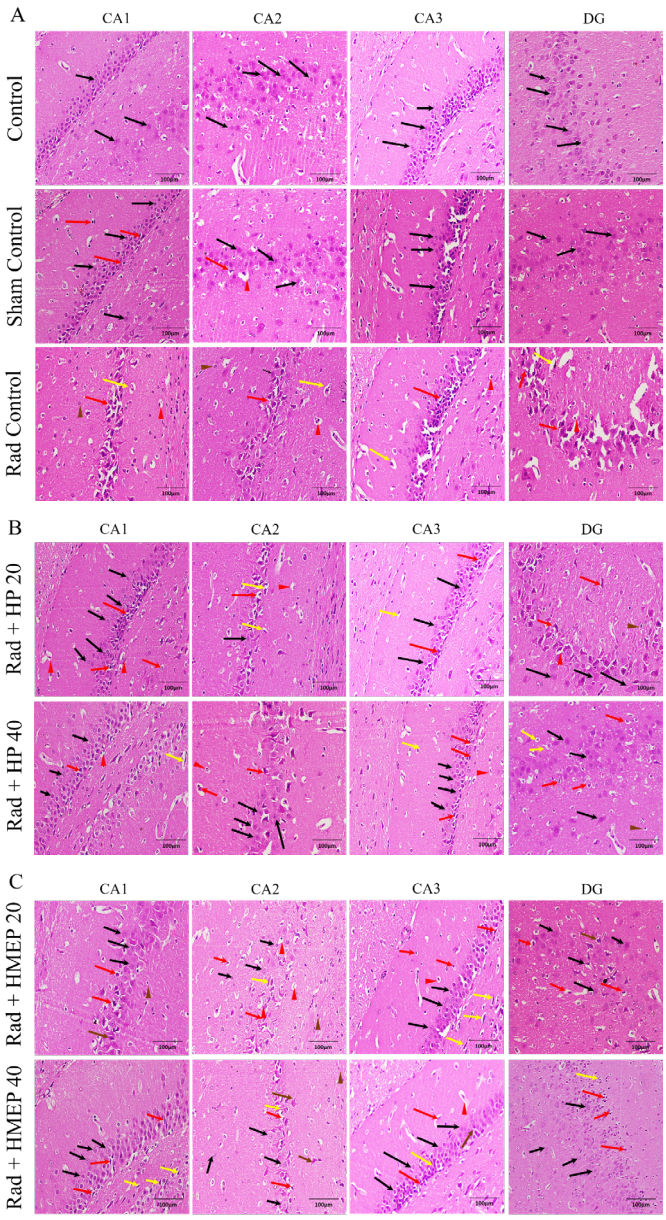

Figure 13. Effect of hispolon compounds on histological changes in the hippocampus (CA1, CA2, CA3, and Dentate Gyrus (DG)) of the rats exposed to RF-EMR. (A) Control groups, (B) Treatment with HP 20 and 40, (C) Treatment with HMEP 20 and 40. Black arrow: Normal neuronal cells, red arrow: necrotic neurons, brown arrow: darkly stained pyramidal cells, yellow arrow: pyknotic, red arrowhead: vacuolization, brown arrowhead: inflammatory cells, green arrow: neurofibrillary tangles. Rad, radiation; HP, hispolon pyrazole; HMEP, hispolon monomethyl ethyl pyrazole.

observing a drop in GSH levels in the brains of rats in response to mobile radiation exposure. It agreed with the outcomes of a report when the rats were subjected to mobile phone radiation (GSM; 900 MHz; SAR-0.9 W/kg) at a rate of 4 hours per day for 15 days; they discovered a significant drop in the GSH levels in the brain (72). Also, rats exposed to mobile phone radiation (GSM; 1800 MHz; SAR-0.6 W/kg) at a rate of 2 hours per day for three months had significantly lower levels of GSH in their hippocampus and cerebellum (35).

SOD deactivates superoxide radicals that are already present while also shielding cells from their damaging effects (73). The current study found that SOD levels were much lower in regions of the brain, which is consistent with many other studies that found that SOD levels were significantly lower in areas of the brain such as the cerebral cortex, hippocampus, and cerebellum, which caused ROS levels to rise (35,72,74). MDA, a marker of lipid peroxidation previously described in brain cells, showed a comparable manifestation of increased free radicals in EMR-exposed rats. MDA is one of the by-products of polyunsaturated fatty acid peroxidation in cells (MDA). In the current investigation, MDA levels were considerably higher in the exposed animals than in the control animals. MDA is overproduced as a result of an increase in free radicals. MDA levels are frequently used to evaluate the antioxidant status and oxidative stress (75). Researchers found that MDA levels were reduced but not significantly (19), possibly as a result of oxidation in some cellular structures in brain tissue at the start of the study, leading to resistance against oxidative attacks at the end of the study, but there was a significant increase in the exposed group when compared to control groups (35,72).

Additionally, it was seen that the CAT levels had fallen in the various parts of the brain. According to earlier studies, the drop in its levels causes a rise in free radical levels and accelerates neurodegeneration (19,72). It can be depicted from the current data that HMEP could significantly protect the brain from the oxidative stress developed from mobile phone radiation. The same could be seen in several studies where hispolon was protected from oxidative stress (27,31-33).

By studying histopathological changes, redox changes may significantly influence the neurophysiologic modifications seen in the current study. Younger rats' brains showed significant vacuoles, increased eosinophilic plaques, and dark neurons in the cerebral cortex after long-term exposure to cell phone radiation. Long-term mobile phone use causes degenerative alterations in hippocampal neurons, which are indicated by an increase in deformed and darkly colored nuclei and a decrease in the total number of neurons in the hippocampus area. Pyramidal cells with distorted irregular forms and some with strongly discolored nuclei with intranuclear vacuolation also showed signs of degeneration from RF-EMR exposure. Long-term RF-EMR exposure also affects hippocampal morphology, as demonstrated by pathological alterations at light microscopic levels. This study's amplitude of tissue degeneration is comparable with previous studies (35,72,76–79). HP and HMEP at higher doses could protect from the neuronal damage caused by the redox instability caused by mobile phone radiation.

Based on the results, the administration of HP and HMEP helps to improve the behavioral, biochemical, and histological changes caused due to mobile phone radiation. The effects of mobile phone radiation were dramatically altered by pyrazole derivatives, demonstrating their protective function (34). The NH proton in the pyrazole moiety found in the diketo region of hispolon and HME is thought to be responsible for antioxidant activity (33,80).

Conclusion

The available data supports the idea that RF-EMR induces oxidative stress, which is mediated by increased lipid peroxidation, impaired redox metabolism, decreased neurotransmitter activity in the cerebral cortex, striatum, and hippocampus, and neurodegeneration in the brain areas. Later, combining the three causes mentioned above led to the neurobehavioral alterations brought on by mobile phone radiation. In contrast, HP and HMEP reduced the aberrant behaviors in the exposed rats and shielded them from oxidative brain damage by mobile phone radiation. The current data may add value to further research in making these compounds for clinical applications.

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Authors' contributions

All the authors contributed to the study conception and design. VPS contributed for the experimentation, data collection, and result analysis. The first draft was compiled by VPS, and DN improvised the draft. CV commented on the previous manuscript. The final manuscript was read and approved by all authors.

Conflict of interests

The authors declare that there is no conflict of interest.

Ethical considerations

IAEC of Vignan Pharmacy College, Guntur, approved all animal studies (006/IAEC/PhD/VPC/2019).

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