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Original Article

Pharmacology Section

Antioxidant Effect of Levetiracetam and Brivaracetam Acting on Presynaptic SV2A Receptors: An In-vitro Study

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ABSTRACT

Introduction: Epilepsy is a serious psychological condition associated with social stigma, psychiatric co-morbidity, and a high economic burden. It is pertinent to develop novel treatment methods given the ever-evolving nature of the disease. Recently, various newer Antiepileptic Drugs (AEDs) have been under study, among which a newer targeted modality consists of SV2A receptors.

Aim: To compare the antioxidant properties or free radical scavenging activity of the newer heterogeneous AEDs acting on the SV2A receptors, namely Levetiracetam (LEV) and Brivaracetam (BRV).

Materials and Methods: This in-vitro study was conducted at Department of Pharmacology, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India from July 2021 to August 2021. The study evaluated the antioxidant properties of LEV and BRV using various assays such as the Ferric Reducing Antioxidant Power (FRAP) test, DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, Nitric Oxide (NO) radical scavenging assay, Superoxide Dismutase activity (SOD), and catalase activity. The obtained results were expressed using specific formulas related to the absorbance of the respective chemicals, along with the percentage of inhibition, and have been tabulated in Microsoft excel Sheet Version 16.16.27. Half-maximal Inhibitory Concentration (IC50) values were calculated using scatter plot graphs.

Results: The FRAP values for LEV were reported to be significantly higher compared to BRV at concentrations of 20, 40, 60, 80 µg/mL, while at 100 µg/mL, BRV showed better ferric reducing activity. Although both drugs exhibited antioxidant activity, the results clearly identified LEV as a better NO radical scavenger, with a percentage of inhibition reaching up to 80.52%. During the SOD assay, the percentage inhibition of superoxide generation by LEV was found to be 99.02%, while that of BRV was 97.07% at a concentration of 100 µg/mL. LEV (0.573) also showed a higher degradation of H_2O_2 per minute than BRV (0.065) at a concentration of 100 µg/mL.

Conclusion: The results showed that both drugs, BRV and LEV, exhibited significant antioxidant capacity. However, LEV demonstrated increased antioxidant potency and efficacy compared to BRV.

Keywords: Antiepileptic drugs, Ferric reducing antioxidant power assay, Nitric oxide assay, Oxidative stress, Reactive oxygen species reduction

INTRODUCTION

Epilepsy is a chronic non communicable brain disorder that affects people of all ages. A 2017 meta-analysis revealed that the overall incidence of epilepsy is 67.77 per 100,000 persons. Most drugs targeting epilepsy are ion channel blockers. It is estimated that 5 million people are diagnosed with epilepsy annually worldwide [1]. Recurrent seizures stem from hyperexcitation of specific groups of neurons, leading to various transient clinical signs and laboratory findings [2].

Epileptic seizures can occur due to neuronal cell death or as a consequence thereof. Several contributing factors include genetic components, the extent of excitotoxicity due to glutamate, which ultimately leads to disruptions in intracellular electrolyte metabolism, increased cytokine concentration, and heightened oxidative stress [3]. Oxidative stress occurs due to an imbalance between the body's antioxidant defense system and free radical production. It remains an important mechanism that plays a role in the aetiology of seizure-induced neuronal death. The most perilous effect of free radicals is lipid peroxidation, leading to the disruption of cell membranes [4].

The human brain, utilising the maximum amount of oxygen compared to other bodily organs, is particularly susceptible to oxidative stress. It contains high concentrations of polyunsaturated fatty acids that are prone to lipid peroxidation and low levels of antioxidant enzymes. Additionally, owing to the high aerobic metabolic rate, the brain itself produces elevated amounts of Reactive Oxygen Species (ROS) such as superoxide, hydroxyl radical, and hydrogen peroxide [5].

LEV and BRV belong to a group of pyrrolidone compounds derived from piracetam, binding at the presynaptic SV2A receptor site, representing a group of AEDs with a distinctive mode of action [6,7]. In the current treatment method, drugs having combined antiepileptic and antioxidant actions are limited [2,8]. Older generation antiepileptic drugs have been shown to increase the oxidant load, leading to an elevated damage rate; however, a few clinical studies have hypothesised probable neuroprotective effects of LEV in neurodegenerative diseases [9-11]. The present study is the first to evaluate the antioxidant effects of the above-mentioned AEDs using an in-vitro model, which should be further validated in an in-vivo model.

The current study aimed to evaluate the antioxidant properties of two newer generation AEDs, LEV and BRV, with the help of an in-vitro model. The experiment was designed to assess the antioxidant properties of these drugs by comparing their antioxidant efficacy not only with a standard but also by analysing and establishing the superior antioxidant agent among the two novel AEDs.

MATERIALS AND METHODS

It is an in-vitro study model using standard assays under Good Laboratory Practice (GLP). The study was carried out at Department of Pharmacology, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India for a period of two

months-July 2021 to August 2021. The study was conducted after obtaining Institutional Ethics Committee approval (REF:CSP/21/JAN/89/54).

Study Procedure

Drugs and chemicals: LEV and BRV-4 tablets of 250 mg each were obtained from Intas. These tablets were dissolved in buffered saline and sonicated for 10 minutes. This was followed by centrifugation and filtration, and then serial dilution. Finally, these were tested in various concentrations of -20, 40, 60, 80, 100 µg/mL.

All the reagents and other chemicals used in the present study were of analytical grade. The chemicals DPPH (1,1-diphenyl-2-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-S-triazine), 2-thiobarbituric acid, ascorbic acid, and Folin-Ciocalteu reagent were obtained from Sigma Chemical Co., 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) and Trolox (6-hydroxy-2,5,7,8-tetramethyl chromane 2-carboxylic acid) were obtained from Sigma Aldrich Chemicals Co., Mumbai, India.

1. **FRAP Assay: Ferric Reducing Antioxidant Power (FRAP):** The method is based on the principle of electron donating antioxidants that lead to the reduction of Fe3+ TPTZ complex (colourless complex) to Fe2+-tripyridyltriazine (a blue-coloured complex) at a low pH [12]. The next step is to observe and measure the change in absorbance at 593 nm. The FRAP reagent was prepared by mixing 300 mM acetate buffer, 10 mL TPTZ in 40 mM HCl, and 20 mM FeCl3.6H_2O in the proportion of 10:1:1 at 37°. It was then pipetted using a 1-5 mL variable micropipette (3.995 mL) and mixed thoroughly with various concentrations (20 mg/mL, 40 mg/mL, 60 mg/mL, 80 mg/mL, 100 mg/mL) of test samples. The ferric tripyridyl triazine (Fe3+ TPTZ) complex was reduced to the ferrous (Fe2+) form, and a profound blue-coloured complex was observed. The absorbance at 593 nm was further recorded against a reagent blank (3.995 mL FRAP reagent + 5 µL distilled water) spectrophotometrically following the procedure of Benzie and Strain after 30 min incubation at 37° [10]. All the determinations were performed in triplicates. After plotting the absorbance at 593 nm versus different concentrations of $FeSO_4$, a calibration curve was created. The concentrations of $FeSO_4$ were also compared against various concentrations of the standard antioxidant Ascorbic acid. The FRAP values were finally obtained after contrasting the absorbance change in the test mixture against those observed from increasing concentrations of Fe3+. These values are expressed here as mg of Ascorbic acid equivalent per gram of the sample. The FRAP values of test samples were calculated from their absorbance readings in relation to the absorbance reading of the standard according to the following formula [13].

$$\text{FRAP Value } (\mu mol/L) = \frac{4' \text{ Absorbance at 590 nm of test sample reaction mixture}}{4' \text{ Absorbance at 590 nm of } Fe^{2+} \text{ STANDARD reaction mixture}} \times Fe^{2+} \text{ Standard concentration}$$

2. **Reduction of DPPH Assay: DPPH (1,1-diphenyl-2-picrylhydrazyl) Assay:** The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay method is based on the reduction of a stable free radical, DPPH. It is a rapid, straightforward, inexpensive method to assess the ability of drugs and compounds to act as free radical scavengers or hydrogen donors while evaluating their antioxidant activity. A maximum absorption at 517 nm (purple colour) is recorded with the free radical DPPH, which contains an odd electron. The absorbance is decreased from DPPH when it reacts with antioxidants and is reduced to DPPH-H. The radical to the DPPH-H form then results in the decolourisation of the assay to yellow. An increase in the decolourisation signifies intense reducing ability.

   The DPPH solution was prepared by dissolving 4 mg of DPPH in 50 mL of methanol. The standard, Butylated Hydroxytoluene (BHT) solution, was prepared by mixing 10 mg of BHT in 10 mL of methanol.

   The test drugs, LEV and BRV, were tested in concentrations of 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, 100 µg/mL. An aliquot of 3.7 mL of absolute methanol was taken in all test tubes, and 3.8 mL of absolute methanol was added to the blank, as described by Molyneux P [14]. The scavenging effect of LEV and BRV on the DPPH radical was calculated using the following formula [15].

Calculation:

$$\% \text{ Antioxidant activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test})}{(\text{Absorbance at blank})} \times 100$$

3. **Nitric Oxide (NO) Radical Scavenging Assay:** The activity of this assay was prepared as per Kumar S et al., who described the preparation of the reagent to measure the NO radical scavenging activity of methanolic fruit extracts, and a similar procedure was followed in the present study [16]. The NO radical scavenging activity of LEV and BRV on the DPPH radical was calculated using the following formula [17].

Formula:

$$\% \text{ scavanging} = \frac{\text{OD of control} - \text{OD of Test}}{\text{OD of control}} \times 100$$

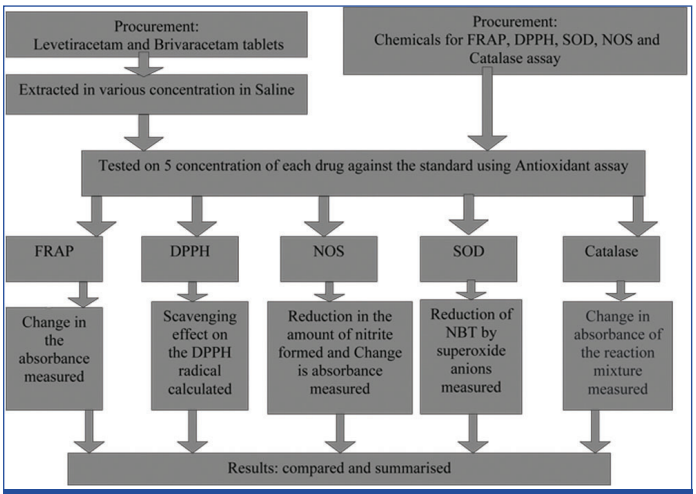
4. **Assay of Superoxide Dismutase (SOD):** SOD is an important antioxidant enzyme that plays a critical role in protecting cells from oxidative stress. An assay for SOD activity can be used to measure the enzyme's ability to convert superoxide anions into Hydrogen Peroxide (H_2O_2) and Oxygen (O_2). One commonly used assay for SOD activity is the Nitro Blue Tetrazolium (NBT) reduction assay. In this assay, SOD activity is measured by monitoring the reduction of NBT by superoxide anions. The assay mixture contained 1.8 mL of Sodium pyrophosphate buffer, 0.1 mL of PMS, 0.3 mL of NBT, 0.6 mL of the enzyme preparation, and water in a total volume of 2.8 mL mixed with the sample according to the modified spectrophotometric assay model described by Kakkar P et al., [18]. The sample is exposed to light, which then generates superoxide anions. The superoxide anions react with NBT, reducing the blue formazan dye. SOD converts superoxide anions to H_2O_2, which does not react with NBT, resulting in less blue formazan dye being formed. The amount of blue formazan dye formed is measured spectrophotometrically at 560 nm, and the decrease in absorbance due to SOD activity is used to calculate the compound's activity [19].

   Calculation:

   $\% \text{ INHIBITION} = (A_{560\,nm} \text{ of blank} - A_{560\,nm} \text{ of test}) / A_{560\,nm} \text{ of blank} \times 100$

5. **Assay of Catalase:** The activity of Catalase was determined as described by Sinha AK [20]. Catalase is a ubiquitous antioxidant enzyme that degrades hydrogen peroxide into water and oxygen, eventually protecting cells from oxidative damage. The catalase assay is a laboratory technique used to measure the activity of the enzyme in a sample. The steps involve first preparing the sample, and then the reaction mixture is prepared by mixing the sample with a substrate solution containing hydrogen peroxide. The mixture is then incubated for a specific period of time, during which catalase in the sample reacts with the hydrogen peroxide and produces oxygen and water. The amount of oxygen produced in the reaction is measured calorimetrically using a suitable reagent. The catalase activity is calculated based on the change in

absorbance of the reaction mixture over time. An overview of the study flowchart is given in [Table/Fig-1].



[Table/Fig-1]: Overview of the study flowchart.

STATISTICAL ANALYSIS

Spectrophotometry using UV probe software is used to gather the data. The obtained results are expressed using specific formulas regarding the absorbance of the concerned chemical, along with the percentage of inhibition, and have been tabulated in Microsoft excel sheet version 16.16.27. Half-maximal Inhibitory Concentration (IC50) values are calculated using a scatterplot graph.

RESULTS

1. **FRAP Assay:** The reducing ability of the drugs was found to be in the range of 1298 µmol/L to 801.11 µmol/L. The FRAP values for LEV were recorded at an elevated level compared to BRV at concentrations of 20, 40, 60, 80 µg/mL. At these concentrations, both BRV and LEV showed superior ferric reducing potential than standard ascorbic acid [Table/Fig-2]. The results suggested that LEV has a consequential ability to react with free radicals, altering them into more stable non reactive species and finally terminating the radical chain reaction. The IC50 values of BRV and LEV were noted as 251.13 µg/mL and 117.92 µg/mL, respectively.
2. **Reduction of DPPH assay:** There was a dose-dependent increase in antioxidant potential of both drugs, similar to that of standard BHT [Table/Fig-3].

S. No.	Concentration (µg/mL)	FRAP value (µmol/L)		
		Standard: Ascorbic acid	Sample: Brivaracetam	Sample: Levetiracetam
1.	20	376.66	801.11	843.33
2.	40	584.44	851.11	924.44
3.	60	783.33	895.55	1001.11
4.	80	1107.77	973.33	1124.44
5.	100	1308.88	1015.55	1298.88

[Table/Fig-2]: FRAP values of standard compared to BRV and LEV.

S. No.	Concentration (µg/mL)	% DPPH Activity		
		Standard:BHT (Butylated Hydroxytoluene)	Sample: Brivaracetam	Sample: Levetiracetam
1.	20	75.30	74.29	75.80
2.	40	77.20	75.90	77.81
3.	60	78.71	78.71	79.51
4.	80	80.02	80.32	81.92
5.	100	82.32	82.32	82.93

[Table/Fig-3]: DPPH activity of standard and sample.
Control- 0.996

IC50 values of BRV and LEV were recorded as 24.475 µg/mL and 23.719 µg/mL, respectively [Table/Fig-4]. These results indicate that BRV and LEV have a marked effect on scavenging free radicals, especially at higher doses.

IC50 value	Values
IC50 Value (µg/mL): Brivaracetam	24.475
IC50 Value (µg/mL): Levetiracetam	23.719

[Table/Fig-4]: IC50 values of BRV and LEV with respect to DPPH activity.

3. **Nitric Oxide (NO) radical scavenging assay:** Though BRV showed potent antioxidant capacity, the IC50 value remained at 9.076 µg/mL, with its inhibition percentage reaching up to a maximum of 79.20% [Table/Fig-5]. The results distinctly identified LEV as a better NO radical scavenger, where the percentage of inhibition reached up to 80.52% with an IC50 value of 8.432 µg/mL.

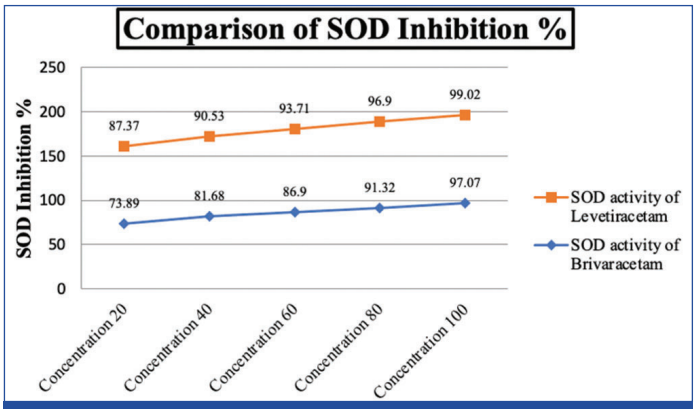
S. No.	Concentration (µg/mL)	NO_2 Inhibition % of Standard	NO_2 Inhibition % Brivaracetam	NO_2 Inhibition % Levetiracetam
1.	20	32.47	70.95	71.61
2.	40	36.77	72.93	73.59
3.	60	43.44	75.24	75.90
4.	80	51.82	77.22	78.54
5.	100	60.64	79.20	80.52

[Table/Fig-5]: NO_2 inhibition % of standard and samples.
Control: 0.465

4. **Assay of Superoxide Dismutase (SOD):** The superoxide scavenging activity of both drugs was noted to be significant in contrast to ascorbic acid used as a standard [Table/Fig-6]. The percentage inhibition of superoxide generation by LEV was found to be 99.02%, while that of BRV was at 97.07% at a concentration of 100 µg/mL [Table/Fig-7]. These results indicate that LEV has a more potent effect of scavenging the free radicals, with an IC50 value of 11.66 µg/mL, while that of BRV was noted as 109.19 µg/mL.

S. No.	Concentration (µg/mL)	Standard: Ascorbic acid	SOD (U/mg protein) SOD activity of Brivaracetam	SOD (U/mg protein) SOD activity of Levetiracetam
1.	20	376.66	73.89	87.37
2.	40	584.44	81.68	90.53
3.	60	783.33	86.90	93.71
4.	80	1107.77	91.32	96.90
5.	100	1308.88	97.07	99.02

[Table/Fig-6]: SOD activity of BRV compared to LEV at different concentrations.



[Table/Fig-7]: Superoxide generation inhibition activity of BRV and LEV.

5. **Assay of Catalase:** LEV (0.573) showed higher degradation of H_2O_2/min than BRV (0.065) at a concentration of 100 µg/mL. Catalase activity was expressed as mol of H_2O_2 consumed/min/mg of protein [Table/Fig-8]. The results demonstrated better

hydrogen peroxide scavenging activity of LEV compared to BRV. Considering all the assays, both the drugs BRV and LEV, compared to the standard, exhibited significant antioxidant capacity. However, LEV showed increased antioxidant potency and efficacy than BRV.

S. No.	Concentration (µg/mL)	Catalase (H_2O_2 consumed/min) Brivaracetam	Catalase (H_2O_2 consumed/min) Levetiracetam
1.	20	0.008	0.147
2.	40	0.016	0.286
3.	60	0.024	0.393
4.	80	0.049	0.467
5.	100	0.065	0.573

[Table/Fig-8]: Compared catalase activity of BRV and LEV.

DISCUSSION

The role of oxidative stress in neuronal death is receiving significant interest in epilepsy research. This interest stems from the brain's relatively weak antioxidant defense mechanisms, making it highly vulnerable to oxidative stress due to its high oxygen consumption, accounting for about 20% of the body's total oxygen consumption. Furthermore, the neuronal membrane contains numerous polyunsaturated fatty acids, making it susceptible to lipid peroxidation, a crucial component of oxidative stress [21]. Additionally, the brain harbours substantial amounts of iron and copper, which facilitate the synthesis of hydroxyl radicals [22]. Neuronal cells in the brain are particularly susceptible to oxidative injury, and under pathological conditions, excessive Reactive Oxygen Species (ROS) brought on by altered redox equilibrium lead to oxidative stress, resulting in neuronal death through various mechanisms, including apoptosis, autophagy, necroptosis, pyroptosis, and ferroptosis. Prolonged convulsions induce oxidative stress, contributing to neuronal death. This supports the concept that oxidative stress plays a significant role in seizure-induced neuronal death and/or the onset of epilepsy [23,24].

In 2004, Liang LP and Patel M demonstrated that superoxide radicals initiate chronic mitochondrial oxidative stress, which is sufficient to increase seizure susceptibility due to aging, environmental stimulation, or excitotoxin administration [25]. Another study conducted by Sashindranath M et al., examined early seizure-induced oxidative stress in wild type and SOD2 (-/+) mice using the Rapid Electrical Amygdala Kindling (REAK) model, which does not induce cell death [26]. Additionally, LEV has previously been shown to protect against neurotoxicity induced by oxidative stress in experimental animal seizure models [27,28]. The role of free radicals in seizures has also been supported by the successful use of exogenously administered antioxidants such as vitamin C and vitamin E to protect the brain against seizure-induced damage [29,30].

Pharmacological studies of exogenous substances or therapeutic treatments have, for a number of years, focused on potential interactions with ROS to assess their ability to prevent or limit free radical damage to biological targets [26,31,32]. The second-generation AEDs, BRV and LEV, are novel molecules clearly distinguished from traditional AEDs by their pharmacological properties and mechanism of action. Although the literature suggests that the anticonvulsant effects of AEDs could be directly related to their antioxidant properties, the basis of these properties remains unclear [33]. The results of the present study clearly demonstrate a significant antioxidant effect of both drugs, which increased with dosage when compared to ascorbic acid. Previously reported data and studies have also demonstrated the well-recognised antioxidant effects of vitamin C. This fact suggests that vitamin C acts as a neuroprotective agent capable of reducing behavioural and pro-oxidative changes associated with the development of seizures [30].

In the present study, treatment with BRV and LEV (at all doses tested) revealed increased ferric reducing potential compared to the standard at lower concentrations, which reduced as the concentration of the drugs increased. Regarding the scavenging activity of the DPPH radical, both AEDs demonstrated increased activity at higher concentrations, such as 100 µg/mL. In terms of nitrite-nitrate concentrations, LEV and BRV showed the capacity to reduce the changes induced by heating with an increased inhibition percentage. Subsequently, the SOD activity was recorded at 99.02% at 100 µg/mL for LEV, with a slight difference from BRV at the same concentration. Finally, catalase activity of LEV was significantly higher than BRV at a concentration of 100 µg/mL, with a degradation rate of hydrogen peroxide of 0.573/min.

The existing literature reports that numerous AEDs, especially from the older generation including phenobarbitone, carbamazepine, and valproic acid, produce reactive metabolites. These metabolites can covalently bind to different endogenous macromolecules, increasing the formation of ROS and inducing oxidative damage and toxicity [31,34]. Martinc B et al., evaluated the influence of older AEDs using a meta-analysis, showing increased or inconclusive Malondialdehyde (MDA) levels in the group treated with older generation AEDs [2]. Similarly, multiple studies have confirmed the theory of increased lipid peroxidation in conventional AEDs like phenobarbitone, phenytoin, and carbamazepine [35,36]. However, in the present study, results demonstrated significant antioxidant and free radical scavenging activity of LEV and BRV at certain concentrations. Additionally, when comparing the two drugs, LEV showed higher potency and efficacy when tested in-vitro using various assays.

Previously, a few studies have revealed a novel spectrum of activity in experimental seizure models [37-39]. To date, the mechanisms underlying the neuroprotective effect are not well understood. Some AEDs, such as phenytoin, have been observed to reduce oxidative stress by increasing Glutathione (GSH) reductase activity [8]. Considering the findings given by Marini H et al., of blunting brain MDA both in cortex and diencephalon in a kainic acid-induced seizures model due to LEV pretreatment, it is possible to speculate an activity against oxidative stress [40]. Apart from epilepsy, LEV has also shown antioxidant and neuroprotective effects in Alzheimer's disease in a number of animal studies [9]. Similarly, the present findings also support this notion, as the drugs showed the ability to reduce lipid peroxidation and increase NO radical scavenging activity after inducing exogenous oxidative stress.

The conversion of H_2O_2 to H_2O can be achieved by catalase and GSH. As stated earlier, epileptogenesis and episodes of status epilepticus lead to increased production of free radicals while suppressing the brain's ability to activate free radical scavenging mechanisms, leading to cell death [41,42]. However, the present data shows enhanced catalase activity of BRV that is directly proportional to the dosage. Thus, the present study suggests the ferric reducing potential, free radical scavenging, increased catalase activity, and SOD potential of newer AEDs-LEV and BRV. Although a few models have been designed to study the clinical aspect of BRV and LEV, in-vitro results have not been demonstrated previously for BRV, to the best of the present knowledge [11,22,37].

Limitation(s)

In-vitro studies cannot replicate the human internal milieu because various physiological interactions and biochemical reactions are not accounted for. In-vitro studies lack the ability to incorporate xenobiotic metabolism, and there are practical difficulties in translating correct in-vivo doses from in-vitro concentrations. Furthermore, the long-term consequences with the help of in-vivo studies cannot be ascertained.

CONCLUSION(S)

In the present study, both drugs exhibited antioxidant activity, with LEV showing increased antioxidant potency and efficacy compared to BRV, which may suggest a neuroprotective action in addition to their already known mechanisms of action. The authors propose further extended clinical research and in-vitro model designs to gather enough evidence to advocate their use in other neurodegenerative diseases.

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