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Original Article

Pharmacokinetic and pharmacodynamic effects of *Withania somnifera* (L.) Dunal (ashwagandha) and carbamazepine combination in rats

[Efectos farmacocinéticos y farmacodinámicos de la combinación de *Withania somnifera* (L.) Dunal (ashwagandha) y carbamazepina en ratas]

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Abstract

Context: *Withania somnifera*, also known as ashwagandha, has antibacterial, anti-inflammatory, anticonvulsant, and anti-diabetic properties and reduces stress. Carbamazepine is one of the most used antiepileptic medications for treating epilepsy.

Aims: To investigate the pharmacokinetics and pharmacodynamics of carbamazepine versus *W. somnifera* extract combination *in vivo* in healthy rat plasma using high-performance liquid chromatography.

Methods: The carbamazepine levels were analyzed using validated high-performance liquid chromatography. The gamma-aminobutyric acid in the serum was performed using an enzyme-linked immunosorbent assay.

Results: Combining *W. somnifera* and carbamazepine resulted in a significant increase in urine volume. There was a slight increase in brain size. It took two to four hours to reach the maximum concentration, a substantial rise. After treatment with *W. somnifera* root extract, in combination with carbamazepine and carbamazepine alone, the concentrations of the gamma-aminobutyric acid in the serum sample were 596, 452, and 505 ng/mL, respectively. Therefore, the serum rat group that received a combination therapy showed a decrease in the gamma-aminobutyric acid level.

Conclusions: Compared to the combination of carbamazepine and *W. somnifera* extract, the time required to reach the maximum concentrations of both the drug and the extract was significantly increased, and the levels of gamma-aminobutyric acid concentration decreased. This could affect the rate at which the stomach emptied, reduce absorption, or change how the medication was absorbed.

Keywords: ashwagandha; carbamazepine; gamma-aminobutyric acid; high-performance liquid chromatography; *in vivo*; rats.

Resumen

Contexto: *Withania somnifera*, también conocida como ashwagandha, tiene propiedades antibacterianas, antiinflamatorias, anticonvulsivas y antidiabéticas y reduce el estrés. La carbamazepina es uno de los medicamentos antiepilépticos más utilizados para tratar la epilepsia.

Objetivos: Investigar la farmacocinética y farmacodinamia de la combinación de carbamazepina versus extracto de *W. somnifera in vivo* en plasma de rata sana mediante cromatografía líquida de alto rendimiento.

Métodos: Los niveles de carbamazepina se analizaron mediante cromatografía líquida de alto rendimiento validada. El ácido gamma-aminobutírico en el suero se realizó mediante un ensayo inmunoabsorbente ligado a enzimas.

Resultados: La combinación de *W. somnifera* y carbamazepina resultó en un aumento significativo en el volumen de orina. Hubo un ligero aumento en el tamaño del cerebro. Tomó de dos a cuatro horas alcanzar la concentración máxima, un aumento sustancial. Después del tratamiento con extracto de raíz de *W. somnifera*, en combinación con carbamazepina y carbamazepina sola, las concentraciones de ácido gamma-aminobutírico en la muestra de suero fueron de 596, 452 y 505 ng/mL, respectivamente. Por lo tanto, el grupo de ratas de suero que recibió una terapia combinada mostró una disminución en el nivel de ácido gamma-aminobutírico.

Conclusiones: En comparación con la combinación de carbamazepina y extracto de *W. somnifera*, el tiempo necesario para alcanzar las concentraciones máximas tanto del fármaco como del extracto aumentó significativamente, y los niveles de concentración de ácido gamma-aminobutírico disminuyeron. Esto podría afectar la velocidad a la que se vacía el estómago, reducir la absorción o cambiar la forma en que se absorbe el medicamento.

Palabras Clave: ácido gamma-aminobutírico; ashwagandha; carbamazepina; cromatografía líquida de alta resolución; *in vivo*; ratas.

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INTRODUCTION

Natural remedies have historically been utilized in the diagnosis, treatment, and prevention of both medical and psychological disorders (Wangchuk, 2018). Herbal therapies may be capable of resolving many of the issues that conventional drugs are unable to solve (Amle et al., 2022). Plant medicines can be administered alone as a treatment option or in combination with conventional drugs. Herbs are considered natural and safe, with few side effects. At the same time, they include physiologically active ingredients that may interact with medications. Such interactions could be synergistic, additive, or antagonistic (Amadi and Nwachukwu, 2020). That said, drug-herb combinations require careful study.

Ashwagandha, also known as "winter cherry," is the common name of *Withania somnifera* (L.) Dunal (family *Solanaceae*). This is a small shrub that can be found in the Middle East (Abdelwahed et al., 2023). Withanolide and sitoindoside are the active ingredients found in *W. somnifera*. Withanolides, which have analgesic, anti-inflammatory, anti-microbial, anti-cancer, and narcotic activities, are primarily found in the root; both are uncommon. The seeds with the most minor medicinal effects can diuretic, hypnotize, and coagulate milk. The leaves also have analgesic, anti-inflammatory, anti-neoplastic, and narcotic properties (Mikulska et al., 2023).

Herbal-drug interactions are a critical topic to study. Carbamazepine (CBZ) is used to manage epilepsy, and *W. somnifera* is used to reduce stress. The coadministration of both could lead to an interaction that results in therapeutic ruin, toxic results, or no effect (neutral). This study aims to investigate the interactions and their impact by experimenting with pharmacokinetics and pharmacodynamics using rats.

CBZ is the most frequently prescribed drug and primary treatment for focal epilepsy. It acts on voltage-dependent sodium channels (Beydoun et al., 2020). The absorption of CBZ is typically slow, irregular, and unpredictable (Baciewicz, 1986). It is a high-protein-binding drug; 70-80% bind to plasma proteins with slight interindividual variation (Mahmood and Chamberlin, 1998). The majority of the CBZ medication is fully digested in the liver, with only around 5% excreted unaltered (Vidaurre et al., 2017). The principal active metabolite of CBZ is electrophilic and pharmacologically active 10,11-epoxide (CBZE). Reports suggest that CBZE can assist in controlling seizures (Bedada and Neerati, 2018).

This article examines the effects of *W. somnifera* extract on carbamazepine's pharmacokinetics and pharmacodynamics in rat plasma. By measuring serum levels of gamma-aminobutyric acid (GABA), the authors identify the anticonvulsant activators of CBZ and the extract from *W. somnifera*.

MATERIAL AND METHODS

Chemical and reagents

Honeywell supplied the advanced gradient grade of acetonitrile and methanol solvent. Trimethylamine was obtained from TEDIA. Ultra-pure water (free from sodium) was also used. The animal house at the Applied Science Private University donated rats. Dar Al Dawaa Pharmaceutical Company provided carbamazepine (CBZ) raw material. Metronidazole benzoate was donated by Jordan Pharmaceutical Manufacturing (JPM). Standardized *W. somnifera* root extract (5% withanoloids) was purchased from BioenergyTech (Amman, Jordan).

W. somnifera standardized extract (5% withanolides) was administered to rats following the human's recommended daily dose of 1000 mg/day (Elgar, 2021; Raut et al., 2012).

To create a CBZ solution, 30 mg of CBZ were dissolved in 15 mL of sodium-free ultra-pure water. Each rat received a daily dose of 2 mg/mL of CBZ.

To prepare for HPLC analysis, 10 mg of CBZ was dissolved in 50 mL of stock (methanol) as a working solution. The solution was stored at -20°C for further use.

A stock solution of the internal standard (IS) was prepared from 10 mg of metronidazole benzoate in 10 mL of acetonitrile to achieve a 1 mg/mL concentration.

The calibration curve for seven concentrations of CBZ (75, 150, 300, 750, 1500, 3000, and 5000 ng/mL) was prepared from an aliquot of 25 µL of the working solution in 1000 µL of plasma. The other six levels were then prepared in series. The quality control (QC) samples for low (QCL), medium (QCM), and high (QCH) levels were prepared as 200, 2100, and 4000 ng/mL, respectively.

To extract CBZ from a sample, 100 µL of the sample was placed in an Eppendorf vial. An aliquot of 150 µL of the internal standard (8 µg/mL of metronidazole benzoate) was added to the vial. The sample was vortexed for one minute at 1000 rpm. An aliquot of 20 µL of supernatant, produced by centrifuging the

sample at 14000 rpm for 15 minutes, was loaded into the HPLC.

Sample collection

After 24-hour fasting, the retro-orbital sinus of rats was used to obtain the plasma. The collected samples were acquired in EDTA tubes at different times (0.25, 0.5, 1, 2, 3.5, 4.5, 6.5, 8, and 24 h). Every plasma sample was centrifuged at 5000 rpm for 10 minutes before being transferred to another Eppendorf tube and stored at -80°C for later analysis.

Serum samples were collected three weeks after the trials started. Before the collection, serum samples were obtained from rats' retro-orbital sinuses after a 24-hour fasting period. The sample was centrifuged in labeled Eppendorf tubes and kept at -80°C for additional biochemical examination.

Biological assay

Following the completion of the sampling required for the analyses, we weighed the rats to determine the effects of the treatment after administering chemicals. In a metabolic cage, the volume of urine and the quantity of feces were recorded. After dissections, the effects of the treatment on the weight and morphological change of some internal organs, such as the liver, kidneys, and brain, were assessed.

High-performance liquid chromatography (HPLC)

The mobile phase of 55% water containing 1 mL trimethylamine per 1 L and 45% acetonitrile was prepared. A pH of 6.00 was adjusted using 0.01 M phosphoric acid. Base deactivated silica (BDS) C18 reversed-phase column (150 mm × 4.6 mm (I.D.), 5 µm particle size) was used. The autosampler temperature was set to 10°C, and the column temperature was set to 25°C. The sample (20 µL) was injected into the HPLC column. The detection was done at 285 nm. The samples were eluted at a 1.0 mL/min rate, with a retention time of 2.9 min for CBZ and 3.9 min for metronidazole benzoate (internal standard).

The experiment was conducted at the University of Petra Pharmaceutical Center's instrumental laboratory. The study employed HPLC (Finnigan Surveyor) equipped with a UV (ultraviolet)-Vis plus Detector (ChromQuest software). An auto-sampler Plus (automatic sampling system), a solvent supply system pump (LC Pump Plus), and the chromatographic were used. The data were analyzed using a computer system with Windows XP and SP3.

Method validation

A validation procedure was carried out according to the ICH guideline M10 on bioanalytical method validation and study sample analysis (Committee for Medicinal Products for Human Use, 2022). The intra-day precision and accuracy were assessed using QC samples at three concentration levels (200, 2100, and 4000 ng/mL). The QC samples were prepared and analyzed together with the calibration samples. The accuracy was determined as the percentage of 100% - relative error, RE %. The precision was evaluated as a percentage relative standard error (%RSD).

Quantification of gamma-aminobutyric acid

To determine the concentration of GABA in serum rats, a rat gamma-aminobutyric acid (GABA) enzyme-linked immunosorbent assay (ELISA) kit (Sunlong Biotech Company) (Shanghai-China) was used. The sandwich-ELISA method, including a micoELISA strip plate pre-coated with a GABA-specific antibody, was performed.

Pharmacokinetic methodology

The study was given the go-ahead by the University of Petra's Faculty of Pharmacy and Medical Science's Ethical Committee of the High Research Council in Amman, Jordan (4/2022). The rats, both female and male Wister-Albino lab rats, weighed around 150 g in this investigation. Air conditioners were used to keep the rats at 22-24°C under a photoperiod cycle of (12 h of light/ 12 h of dark). Food and water were provided *ad libitum* before blood samples were collected.

All rats were weighed, randomized, and divided into four groups: Group 1 (n = 6) was treated with *W. somnifera* root extract (2 mg/rat), group 2 (n = 6) was treated with CBZ (2 mg/rat) and *W. somnifera* (6 mg/rat) combination therapy, group 3 (n = 6) was treated with CBZ (10 mg/kg) alone, group 4 (n = 3) received no treatment, and all were administered using appropriate oral gavage. The CBZ doses were given based on 800 to 1200 mg/day for adults and children aged above 12 and 400 to 800 mg/kg for children aged 8 to 12 (Gierbolini et al., 2016). Based on this information, the used doses were set for CBZ and *W. somnifera*.

ADME and toxicity

CBZ and withanolide A information was obtained using SwissADME SwissDrugDesign (http://www.swissadme.ch). Gastrointestinal absorption is considered high for CBZ and withanolide A. The results showed that CBZ is a highly P-glycoprotein substrate, and withanolide A is not. CBZ could also inhibit CYP1A2 and CYP2C19, while withanolide A did not. CBZ toxicity can happen if the drug is taken excessively. Therefore, following doctor's prescriptions and doses is very important. As a

result, no toxicity will be detected following the correct instructions. As per the Lipinski (Pfizer) filter, the drug-likeness for CBZ and withanolide A is high.

Statistical approach

Statistical analysis was performed by using Microsoft Excel and Winnonlin V5.2 for pharmacokinetic analysis, including an independent t-test, and using Prism 8 for analysis of ELISA kit result, including the one-way ANOVA test, in which each data point represents the mean ± the standard error of the mean (SEM). A p-value of 0.05 or less was considered significant ($p \leq 0.05$).

RESULTS AND DISCUSSION

HPLC validation

Linearity

A calibration curve was constructed using seven concentrations for the quantitative analysis of CBZ in plasma samples. The coefficient of determination (R^2) was (0.9986) over a 75-5000 ng/mL range, as shown in Fig. 1.

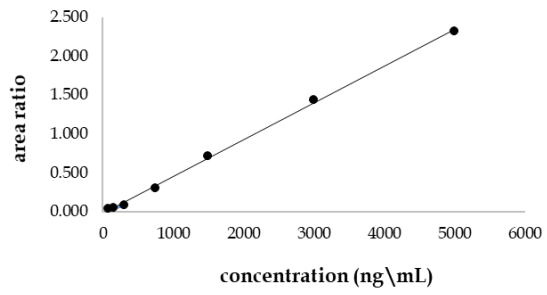

Figure 1. Carbamazepine calibration curve.

Accuracy and precision

The method's precision and accuracy were determined using QC samples of CBZ in the different samples, as illustrated in Table 1. The accuracy percentages for low, medium, and high-quality control samples (100.6, 103.1, and 99.6%, respectively) fell between the authorized ranges from 85% to 115%. The precision was determined. The QCL, QCM, and QCH results were 2.4, 6.8, and 2.8%, respectively.

Pharmacokinetic parameters

The HPLC analysis that follows T_{max} of CBZ plasma concentration was significantly changed ($p=0.0008$) and elevated following CBZ-*W. somnifera* combination therapy, as shown in Fig. 2. It is important to note that T_{max} exhibited a highly significant change ($p=0.008$) with a little increase in plasma concentration. However, this increase was not statistically significant. However, the combination therapy did not affect other pharmacokinetic parameters such as elimination rate constant (K_{el}) and half-life ($p=0.688$). Also, the area under the curve (AUC) did not change significantly ($p=0.494$).

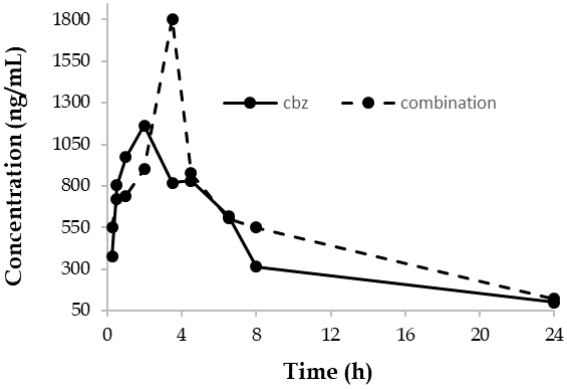

Figure 2. Plasma concentration of CBZ with and without *Withania somnifera*.

Data are shown as mean ± SEM (n = 6).

Interaction between CBZ and *Withania somnifera* root on GABA

A standard curve was constructed for serum samples using six concentrations; R^2 was 0.9919, as shown in Fig. 3. The rats were divided into three collections (n = 6 rats per set). A 10 mg/kg CBZ was administered unaccompanied, CBZ and *W. somnifera* combined therapy, and *W. somnifera* root extract was administered by itself. Serum samples were gathered. The GABA level concentrations were determined using an ELISA kit, as shown in Tables 2-4.

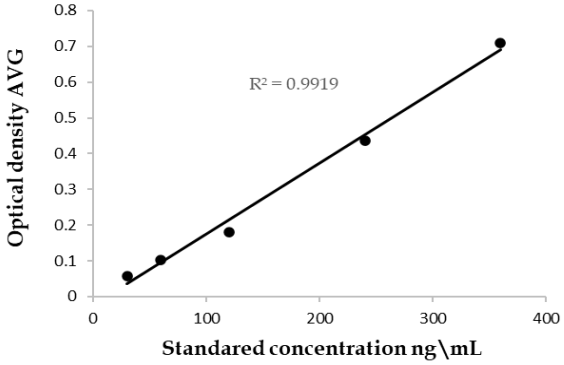

Figure 3. Standard curve of dilution sample of ELISA kit (GABA concentrations).

The concentration of GABA significantly differed ($p=0.05$) between groups treated with CBZ, *W. somnifera* alone, and CBZ combined with *W. somnifera*. In addition, the serum concentration of GABA decreased in the group receiving CBZ and *W. somnifera* combined therapy.

Table 1. Accuracy and precision of the samples of CBZ related to quality controls (QC).

Item	QC-Low (ng/mL)	QC-Medium (ng/mL)	QC-High (ng/mL)
Theoretical	200	2100	4000
Sample1	198	2079	4013
Sample2	207	2113	4008
Sample3	207	2028	4046
Sample4	201	2430	4123
Sample5	196	2233	3813
Sample6	198	2102	3892
Accuracy (%)	100.6	103.1	99.6
Precession (%)	2.4	6.8	2.8

Table 2. Concentrations of GABA in serum sample after being treated (4 hours) with *Withania somnifera.*

Sample	Average optical density	Average-Blank	Concentration (ng\mL)	Average concentration (ng\mL)
G1R1	0.27 ± 0.02	0.21 ± 0.02	599 ± 52	596 ± 52.1
G1R2	0.26 ± 0.02	0.20 ± 0.02	572 ± 52	
G1R3	0.29 ± 0.02	0.23 ± 0.02	644 ± 52	
G1R4	0.25 ± 0.02	0.19 ± 0.02	531 ± 52	
G1R5	0.26 ± 0.02	0.20 ± 0.02	551 ± 52	
G1R6	0.30 ± 0.02	0.24 ± 0.02	680 ± 52	

Data are shown as mean ± SEM (n = 6). G1R1-6: Group number one and rat numbers one to six.

Table 3. Concentrations of GABA in serum sample after being treated with combinational therapy of CBZ with *Withania somnifera* (3 hours and 30 minutes).

Sample	Average optical density	Average-Blank	Concentration (ng\mL)	Average concentration (ng\mL)
G2R1	0.20 ± 0.02	0.14 ± 0.02	405 ± 61	452 ± 60.8
G2R2	0.21 ± 0.02	0.15 ± 0.02	436 ± 61	
G2R3	0.21 ± 0.02	0.15 ± 0.02	415 ± 61	
G2R4	0.20 ± 0.02	0.14 ± 0.02	395 ± 61	
G2R5	0.24 ± 0.02	0.18 ± 0.02	494 ± 61	
G2R6	0.26 ± 0.02	0.20 ± 0.02	568 ± 61	

Data are shown as mean ± SEM (n = 6). G2R1-6: Group number two and rat numbers one to six.

Table 4. Concentrations of GABA in serum sample after being treated with CBZ (1 hour and 33 minutes).

Sample	Average optical density	Average-Blank	Concentration (ng\mL)	Average concentration (ng\mL)
G3R1	0.25 ± 0.01	0.19 ± 0.01	523 ± 38	505 ± 38
G3R2	0.24 ± 0.01	0.18 ± 0.01	510 ± 38	
G3R3	0.24 ± 0.01	0.18 ± 0.01	502 ± 38	
G3R4	0.22 ± 0.01	0.16 ± 0.01	422 ± 38	
G3R5	0.23 ± 0.01	0.17 ± 0.01	485 ± 38	
G3R6	0.26 ± 0.01	0.20 ± 0.01	570 ± 38	

Data are shown as mean ± SEM (n = 6). G3R1-6: Group number three and rat numbers one to six.

On the other hand, those who got *W. somnifera* alone saw a rise in their blood levels of GABA.

Regarding the *in vivo* study, and due to limited study time, the actual effect of *W. somnifera* and CBZ on kidney and liver weight was insignificant; the p-values were 0.62 and 0.19, respectively. Although there was a slight increase in brain weight when the combination was administered compared to the control group, there was no apparent connection to explain this minor change. However, it could be genetic or hereditary factors related to relative body weight. Regarding body weight, no studies have suggested that *W. somnifera* or CBZ may cause weight gain; some studies have suggested the opposite: Patients treated with *W. somnifera* root extract experienced a statistically significant reduction in body weight and body mass index (BMI) in long-term treatment (Choudhary et al., 2017). For CBZ, a study showed that after the 45th day of CBZ medication, there was a statistically significant decrease in body weight compared to the control group (Santhrani et al., 2013). Still, in our short-term study, our finding shows a non-significant change in body weight ($p>0.05$). For feces amount, there was no significant change in the p-value when *W. somnifera* or CBZ was given alone ($p=0.36$) or in combination therapy ($p=0.17$) compared to the control, even though diarrhea is a modest side effect of (CBZ) treatment (Iyer et al., 1992). Previous research on *W. somnifera* found that excessively high doses can produce stomach distress and diarrhea (Chandrasekhar et al., 2012).

There was a significant difference between the urine output groups, mainly when *W. somnifera* and CBZ were administered compared to the control group, as the volume was nearly three times higher. When the CBZ was administered, the amount increased for the control but was reduced for the *W. somnifera*. This differs from what earlier research indicated because CBZ enhanced urine osmolality while decreasing urine volume (Al-Waili et al., 2006), causing CBZ to increase antidiuretic hormone (ADH), which will cause inappropriate sensitivity to renal tubules. Additionally, the formation of nitric oxide, which blocks ADH, is significantly reduced by CBZ (Wang et al., 2014). Whereas *W. somnifera* has a diuretic effect where withaferin A found in *W. somnifera* extract is inducible of nitric oxidase synthase, enhancing NO level (Pathak et al., 2021). This could explain what happened to the urine volume when the combination was given.

For pharmacokinetics parameters, there was no significant change in the apparent half-life of the CBZ medication or in the elimination when combined with *W. somnifera* ($p=0.68$). For metabolism, CYP3A4 is a significant enzyme that converts CBZ to its active metabolite in the liver (Schwarz et al., 2021). On the other hand, *W. somnifera* is considered safe to co-administer with the substrates of CYP3A4 (Kasarla et al., 2022). The only parameter in the pharmacokinetic profile that has a significant change is the time to reach peak plasma concentration (T_{max}), which was increased by two hours, and the $p=0.0008$. Also, the concentration was increased slightly but not significantly.

Previous studies showed that *W. somnifera* reduces gastric emptying due to the duration of food passing through the gastrointestinal tract (Maji and Banerji, 2016). These factors could affect gastric empties in rats and possibly cause them to absorb the drug more slowly. In addition, protein binding could be employed to delay absorption and have an impact on distribution because high protein binding medications produce pharmacokinetic and pharmacodynamics alliteration and cause a high volume of distribution (Ulldemolins et al., 2011), given the fact that the CBZ is highly linked to plasma protein (Charlier et al., 2021). Previous studies have demonstrated a high binding of withanolides to serum albumin (Dubey et al., 2018). *W. somnifera* may slow the rate of CBZ distribution, which could delay reaching high concentration.

The delay in reaching the highest concentration could also be explained by the GABA level results using the ELISA kit. It is well known that one of the mechanisms of action of carbamazepine is to increase GABA levels. It is a Na^+ and GABA channel inhibitor (Charlier et al., 2021). However, using the *W. somnifera* root extract increases the levels of GABA more than the drug. For several decades, *W. somnifera* root extract has been demonstrated to have GABA-mimetic action (Candelario et al., 2015). A previous study indicated that *W. somnifera* components are GABAA and GABA1 receptor agonists (Savage et al., 2018). We investigated its effect on GABA levels *in vivo* using rat serum, and when they were given as a combination, there was a significant change in the concentration; as the concentration of GABA level decreased, the $p<0.05$.

Future studies should find patients consuming the CBZ with *W. somnifera*. Later, experiments should be designed to study human herbal-drug interaction and compare it to scientific findings in rats.

CONCLUSION

This study investigated possible interactions between *Withania somnifera* extract and carbamazepine in rat plasma to help researchers and medical profes-

sionals with combinational therapy (using both conventional and alternative drugs). Additionally, by measuring the level of GABA in serum, the study determined the anticonvulsant activities of both *Withania somnifera* and carbamazepine alone and in combination. The co-administering of *Withania somnifera* root extract and carbamazepine prolonged the time it took for carbamazepine to reach its maximum concentration.

The GABA concentration dropped, suggesting they might have a competitive inhibition effect on the same receptor. These effects were brought about by *Withania somnifera's* phytochemical compounds, which raise GABA levels so that they can be used alone to treat epilepsy. However, they may also impact other medications that mimic GABA. Because of these factors, medical practitioners should know about the interactions between CBZ and *Withania somnifera* root extract and advise against taking them simultaneously.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Concepts or ideas								x
Design		x	x	x				x
Definition of intellectual content		x	x	x			x	x
Literature search	x		x		x	x		x
Experimental studies	x				x	x		x
Data acquisition	x				x	x		
Data analysis	x	x	x	x	x	x	x	x
Statistical analysis		x		x				
Manuscript preparation	x	x	x	x	x	x	x	x
Manuscript editing	x	x	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x	x	x

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