RESEARCH ARTICLE

The Effect of Long-term Administration of *Withania Somnifera* on the Expression of Brain P-glycoprotein Transporter Gene (ABCB1a)

Esraa S. Hassan,[1] Selman M. Selman,[1] Alaa J. Mahrath,[2] Zahraa S. Mangi[1]

[1]*Department of Pharmacology, College of Medicine, University of Babylon, Hillah, Iraq*
[2]*Department of chemistry and Biochemistry, College of Medicine, University of Babylon, Hillah, Iraq*

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ABSTRACT

Depression is among the most serious medical conditions worldwide. Increasing incidence of treatment-resistant depression is a serious problem nowadays. Since extensive P-glycoprotein (P-gp) expression in the blood-brain barrier is already identified as a major obstacle to the effective treatment of several central nervous system (CNS) disorders including depression. This study aims to show the effect of long-term administration of the herbal plant *Withania somnifera* on the expression of P-gp gene and hence its effect on brain paroxetine concentration. Sixty male Albino rats were utilized and allocated into 6 groups: group 1 received no treatment and saved as control, group 2 received only a single dose of 10 mg/kg paroxetine, group 3 received 40 mg/kg of verapamil for 10 days and a single dose of 10 mg/kg paroxetine and groups 4, 5, and 6 received (50, 100, and 200) mg/kg of *W. somnifera* for 21 days respectively and a single dose of 10 mg/kg paroxetine on day 22, all were given treatment orally by using gavage tube. The influence of *W. somnifera* on the paroxetine antidepressant activity was evaluated in forced swimming test. Then, the expression of the ABCB1a gene, which encodes P-gp, in the brain was measured using a real-time polymerase chain reaction. As a result, the mean immobility time and gene fold expression of the groups that received *W. somnifera* significantly decreased as compared to the control group. So, twenty-one days of administration of 50, 100 and, 200 mg/kg *W. somnifera* led to a noteworthy decrease in the mean immobility time in forced swimming test and a significant inhibition of P-gp gene.

Keywords: ABCB1 gene, Forced swimming test, p-glycoprotein, Paroxetine, Real-time polymerase chain reaction (RT-PCR) *Withania somnifera*.

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INTRODUCTION

Depression is a widely disseminated affecting disorder; which can varry from a mild illness, with nearly normal life, to extreme depression with delusions and hallucinations. Depression is a leading cause of worldwide disability and death.[1] Treatment-resistant depression is a significant challenging issue in psychiatry, with up to 50–60% of patients suffering from depression failing to respond adequately to antidepressant drugs. The multidrug resistance transporter P-gp, which is expressed in the blood-brain barrier (BBB), appears to play a key role in the brain distribution of some, but not all, antidepressants, according to preclinical evidence.[2] P-gp is a clinically significant and well-studied BBB transporter. Several antidepressants' brain concentrations are dependent on P-gp, according to animal models. However, biochemical properties that could allow for the evaluation of P-gp pharmacodynamical influence have yet to be discovered, necessitating extensive experimental testing of every new drug to establish its P-gp substrate status.[3] ABC transporter P-gp which is encoded by ABCB1 gene which is found in humans, while the ABCB1a and ABCB1b genes are found in rodents.[4] In this study, the investigation focused merely on the the ABCB1a isoform's contribution, which is recognized to make a significant contribution to the P-gp CNS-protective role.[5] According to studies, P-gp efflux at the BBB may play a role in the high rate of failure of antidepressant drugs by preventing some patients from reaching therapeutic brain levels.[2] Higher P-gp expression at the BBB and accordingly a more active P-gp pump is thought to lead to lower brain concentrations of P-gp substrate antidepressants because more of the drug is transported back into the bloodstream. On the other hand, lower P-gp expression or reduced P-gp activity is assumed to facilitate the entrance of P-gp antidepressant drugs into the brain which results in higher drug accumulation in the brain.[6] *W. somnifera* also known as Ashwagandha, or Indian Winter is a valuable medicinal plant used since ancient times.[7] Parts of W. somnifera like root, leaf, fruit, and seed have many bioactive compounds and were

**Author for Correspondence: esraas.hassan91@gmail.com*

shown in previous studies to have anticancer, anti-arthritic, antioxidant, anti-diabetic anti-ischemic, antimicrobial, antiepileptic, anti-stress, cardioprotective, anti-inflammatory, and neuroprotective properties.[8] And since *W. somnifera* is known as Indian ginseng, the present study aims to evaluate the effects of long-term administration of *W.somnifera* on the mean immobility time and the brain expression of P-gp gene.

MATERIALS AND METHODS

In this experiment sixty male, albino rats were used. With weights ranging from 340–200 grams. The rats were kept in the Animal House of the College of Medicine, University of Babylon, and kept on 25°C supplied with water and food. The animals were adapted for three weeks and then randomly divided into six groups according to the experiment protocol, approved by the committee of publication ethics at the College of Medicine, University of Babylon, Iraq. Each rat was used only once.

Drug Administration

The dried root of W. somnifera was obtained from Lamar Natural Pvt. Ltd., Mumbai, India, in July 2020. The dried root was crushed by using a mechanical grinder (silver crest) into a fine powder. Then 500 mL of 80% ethanol was used to extract 50 g of the powder at 60°C for 5 hours using a soxhlet extraction apparatus, and the extract was filtered using a filter paper and then dried in a microwave oven to allow evaporation of ethanol. Then 20 mL distilled water was used to dissolve every 1 gm of the extract, so every 1 mL contain 50 mg of the extract.[9] Paroxetine standard powder (10 mg/kg from Shenzhen Dajing Chemical Co., Limited., China) and verapamil (40 mg/kg from Abbott, Turkey) were suspended in distilled water so every 10 mL of the suspension contain 10 mg of the drug. The solutions/suspension and the plant suspension were prepared before the experiments and administered orally by an oral gavage tube.

General Experimental Procedure

Sixty male Albino rats have been divided into six groups randomly, 10 rats for each group. These groups are, Group 1: received no treatment (negative control). Group 2: Each rat received a single dose of paroxetine (10 mg/kg).[10] Group 3: each rat received verapamil (40 mg/kg) for 10 days, then on the 11th day receive a single dose of paroxetine (10 mg/kg).[11] Group 4: each rat received *W. somnifera* (50mg/kg) for 21 days, then on day 22 received a single dose of paroxetine (10 mg/kg).[12] Group 5: each rat received *W. somnifera* (100 mg/kg) for 21 days, then on day 22 received a single dose of paroxetine (10 mg/kg).[9] Group 6: each rat received *W. somnifera* (200 mg/kg) for 21 days, then on day 22 received a single dose of paroxetine 10 mg/kg. All these groups received treatment orally by using a gavage tube.[13] Twenty-four hours after the last dose, a swimming test was performed for each rat, and a video camera recorded all behaviors. Then, each rat was sacrificed under anesthesia by using inhaled chloroform.[10]

Forced Swim Test (FST)

A cylindrical glass box (30 cm*30 cm*70 cm) was made by a researcher,[14] in which each rat individually, were forced to swim to a depth of 30 cm. The glass box was filled with tap water (25°C). The animals were individually allowed to swim for 5 minutes—an observer who was blind to the animal groups recorded and assessed the test sessions. During the first 5 minutes of the swimming session, the total duration of immobility was recorded. Swimming time is the time the animal moves around in the water. This behavior tends to reduce over time until the end of the test. While immobility time refers to the amount of time, the animal spends not moving or moves to keep its head above water while not planning to move around the apparatus.[15]

Collection of Brain Tissue Samples

The skin on the head was removed after decapitation, and the muscle and fascia on the dorsal and posterior parts of the skull were teased/scraped away with small surgical scissors. The brain was carefully removed from the bone and rinsed in saline at 4°C before the arachnoid membrane was carefully removed. Then, 100 mg from the tissue was cut off into small pieces by using a blade, put in an Eppendorf tube, and 2 mL of RNA later was added. Then left it at room temperature for 24 hours and frozen after that in (-20°C) until use.

Quantitative Polymerase Chain Reaction

RNA expression was quantified using traditional procedures using real-time quantitative polymerase chain reaction (qPCR) with the SYBR green reporter dye. Template deoxyribonucleic acid (cDNAs) in each sample tested for quantitative expression levels of ABCB1a (Asab2) gene and housekeeping gene (Asact2) and rotor-gene q RT-PCR detection system (QIAGEN/ Germany) were used.

Data Analysis of qRT-PCR

The relative quantification gene expression levels (fold change) (THE 2-ΔΔCT METHOD Using a reference gene) was used to analyze the data from q RT-PCR for target and housekeeping genes.[16] As following:

ΔCt = Avg. gene Ct - Avg. housekeeping Ct
$\Delta\Delta Ct$ = Avg. gene ΔCt - Avg. control ΔCt
$2^{-\Delta\Delta Ct}$ to normalize the gene amount relative to control

Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out by using repeated measure one-way ANOVA and *post hoc* test. Differences were considered statistically significant if the *p*-value is lower than 0.05.

RESULTS

Forced Swim Test (FST)

The immobility times of all groups were significantly decreased (p-value > 0.05) as compared with group 1. In group 5, there was a significant decrease in immobility time as compared with groups 3, 4, and 6 (Figure 1 and Table 1).

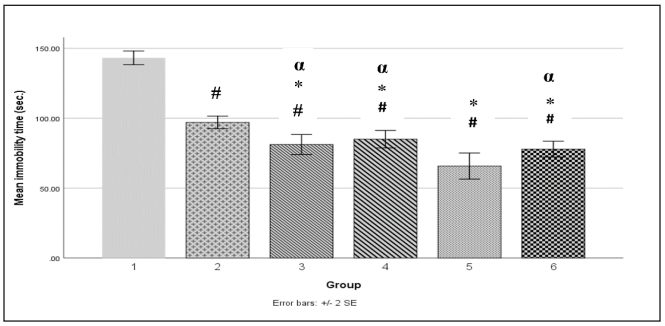

Figure 1: Means of immobility time ± SEM in forced swimming test for all groups.
= significantly decrease (*p*-value <0.05) as compared with group 1.
* = significantly decrease (*p*-value <0.05) as compared with group 2.
α = significantly decrease (*p*-value <0.05) as compared with group 5.

Table 1: Comparison between the mean difference of immobility time (FST) of all groups

	Group 1	*Group 2*	*Group 3*	*Group 4*	*Group 5*	*Group 6*
Group 1	X	–46.2 #	–62.0 #	–58.2 #	–77.4 #	–65.3 #
Group 2	46.2 #	X	–15.8 #	–12.0 #	–31.2 #	–19.1 #
Group 3	62.0 #	15.8 #	X	3.8	–15.4 #	–3.3
Group 4	58.2 #	12.0 #	–3.8	X	–19.2 #	–7.1
Group 5	77.4 #	31.2 #	15.4 #	19.2 #	X	12.1 #
Group 6	65.3 #	19.1 #	3.3	7.1	–12.1 #	X

(p-value <0.05)

Group 1 (control group, untreated), group 2 (treated with a single dose of 10 mg/kg paroxetine), group 3 (treated with 40 mg/kg of verapamil and a single dose of 10 mg/kg paroxetine), group 4 (treated with 50 mg/kg of *W.somnifera* and a single dose of 10 mg/kg paroxetine) and group 5 (treated with 100 mg\kg of *W.somnifera* and a single dose of 10 mg/kg paroxetine) and group 6 (treated with 200 mg/kg of *W. somnifera* and a single dose of 10 mg/kg paroxetine)

No. of rats = 10 for each group using ANOVA and *post hoc* test.

GENE EXPRESSION

ABCB1a Expression

The means fold of ABCB1a expression significantly decreased (*p*-value <0.05) in groups 3, 4, 5, and 6 compared to group 1. Also, in groups 3, 5, and 6, the means fold of ABCB1a expression was significantly decreased (*p*-value < 0.05) as compared with group 2 (Figure 2 and Table 2).

Group 1 (control group, untreated), group 2 (treated with a single dose of 10 mg/kg paroxetine), group 3 (treated with 40 mg/kg of verapamil and a single dose of 10 mg/kg paroxetine), group 4 (treated with 50 mg\kg of *W.somnifera* and a single dose of 10 mg/kg paroxetine) and group 5 (treated with 100 mg\kg of *W.somnifera* and a single dose of 10 mg\kg paroxetine) and group 6 (treated with 200 mg/kg of W. *somnifera* and a single dose of 10 mg\kg paroxetine)

No. of rats = 10 for each group using ANOVA and *post hoc* test.

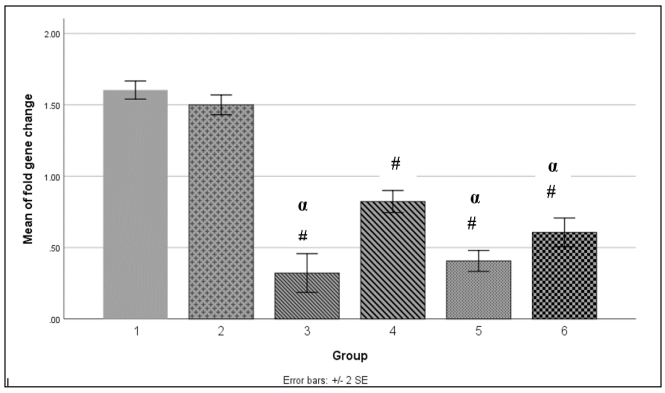

Figure 2: Means ± SEM of ABCB1a fold changes in the brain in all groups.
= significant decrease (p-value <0.05) as compared to group 1 and 2.
α = significant decrease (p-value <0.05) as compared to group 4.

Table 2: A comparison of the means difference of ABCB1a gene expression in brain in all groups

	Group 1	*Group 2*	*Group 3*	*Group 4*	*Group 5*	*Group 6*
Group 1	X	–0.103	–1.282 #	–0.781#	–1.197 #	–0.997 #
Group 2	0.103	X	–1.178#	–0.677 #	–1.093 #	–0.893 #
Group 3	1.282 #	1.178 #	X	0.500 #	0.084	0.284 #
Group 4	0.781#	0.677 #	–.500 #	X	–0.416 #	–0.215 #
Group 5	1.197 #	1.093 #	–0.084	0.416 #	X	0.200 #
Group 6	0.997 #	0.893 #	–0.284 #	0.215 #	–0.200 #	X

(p-value <0.05)

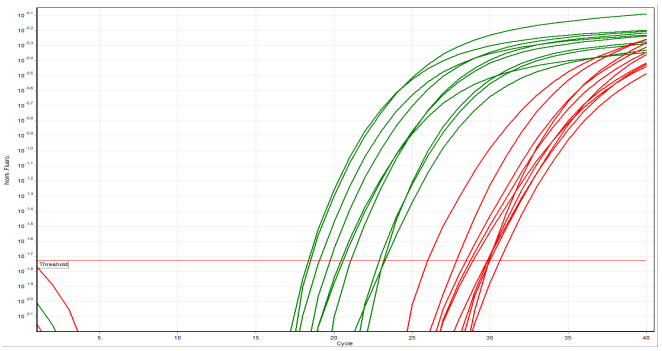

Figure 3: Real-time PCR amplification plot for ABCB1a tissue samples

PCR Amplification Plot

An amplification plot is created when the fluorescent signal from each sample is plotted against the cycle number. Baseline is the initial cycle of RT-PCR during which there is a little change in fluorescent signal. Then comes the threshold, the signal level that statistically represents a significant increase over the measured baseline signal. The threshold cycle (CT) is the number of cycles in which the reaction's fluorescent signal crosses the threshold.

Our study showed a clear difference in threshold cycle numbers (Ct value) between gene groups (the red color) and

the housekeeping gene (which is a reliable internal control gene product for the normalization of expression levels between experiments. Which is used to achieve accurate and reproducible expression profiling of selected genes using RT-PCR) (the green color). No.of rats=10 for each group.

DISCUSSION

Sometimes standard treatments of depression may not help, so other options for treatment-resistant depression should be explored. As the use of herbs as a drug increases, the synthetic drug-herbal interactions become more important and bring more attention to the healthcare professionals to investigate. So in this current study, the effect of the herbal extract *W. somnifera* on the concentration of antidepressant drug (paroxetine) was measured by assessing FST. From the experimental results, the mean immobility time was significantly decreased in all groups compared to the control group, which received no treatment. The group that received 10 mg/kg of paroxetine in asingle dose shows a decrease in mean immobility time since paroxetine is one of the most potent inhibitors of serotonin reuptake of all currently available antidepressants. This effect is reported by Jin *et al.* 2017, who reported thatparoxetine decreased immobility time.[17] The more significant increase in group 3 due to verapamil effect as P-gp inhibitor,[18] which led to increasing paroxetine concentration in the brain, and thus its effect was increased since even though paroxetine is a weak inhibitor, norepinephrine uptake. However, much more potent than other selective serotonine reuptake inhibitors at this site could contribute to its efficacy at higher doses.[19]

The decrease in immobility time in groups receiving 50 mg/kg, 100 mg/kg, and 200 mg/kg of *W. somnifera* for 21 days in all doses plus a single dose of 10 mg/kg of paroxetine. It may happen due to the plant's antidepressant effect possibly mediated through α-adrenoreceptor and an alteration in the main biogenic amines level.[20] The most significant decrease in immobility time in the group that received 100 mg/kg and single-dose 10 mg/kg paroxetine was noted by the shah study. Also, Mandlik and Namdeo, 2021 reported that pre-stress treatment of mice with W. somnifera resulted in a significant reduction in immobility time, demonstrating *W. somnifera*'s anti-stress activity due to its antioxidant properties.[21]

Since P-gp is responsible for the active extrusion of a diverse range of chemical structures that are structurally unrelated,[22] so the widespread expression of P-gp in the BBB has been identified as a significant barrier to effective treatment of many CNS disorders. Notably, changes in the extent of this transporter's expression and/or activity pattern can have a significant impact on the pharmacokinetic profile of drug particles that interact with it, leading to clinical drug-drug interactions.[23]

Because the majority of drugs are made up of tiny molecules which bind to target proteins, inhibiting or activating their biological function. Drugs frequently interact with a variety of proteins, both primary and other proteins (off-targets). To increase medication efficacy and safety, assessments of probable pharmacological effects on single target proteins and many off-target proteins.[24] Sawada *et al.* proposed that if a chemical inhibits a specific protein, the consequent gene expression profile can be linked to the gene expression profile following gene knock-down of the corresponding protein. Similarly, if a substance activates a specific protein, the gene expression profile following chemical treatment of the substance may be linked to the gene expression profile when the protein is overexpressed.

In the present study, the fold gene expression of the ABCB1a was measured for all groups. Based on the results shown in this study, the CT values of groups 3, 4, 5, and 6 were low, and the fold changes of gene expression were significantly down-regulated, while CT values of group 2 were slightly down-regulated as all compared with group 1, which receive no treatment.

There was a significant decrease in this gene in group 3 that received verapamil, an inhibitor of P-gp expression, as noted by Wang *et al*., 2010. They found that verapamil significantly inhibited the expression of P-gp in the lung cell lines that they investigate.[25] Also, this result agrees with the study that shows that when the degree of drug efflux inhibition and multidrug resistance 1 suppression effects are considered, verapamil appears to be a better multidrug resistance modulator than promethazine in doxorubicin-resistant MCF-7 cells.[26] The groups that received (50, 100, 200) mg\kg of W.somnifera exhibited a significant decrease in the fold gene expression compared to the control group, with maximum decrease occurred in group 5 that received 100 mg/kg of the plant. Also, there was a slight decrease in the gene fold in the group that received a single dose of paroxetine as this drug have a slight ability to reduce P-glycoprotein activity,[27] since paroxetine is both a substrate and inhibitor of P-gp,[28] but the decreased gene fold in this group was not significant since the animals received only a single dose of paroxetine.

The RT-PCR results, which indicate that W.somnifera has a significant P-gp inhibition effect, explain the significant increase in paroxetine concentration in the brain measured by HPLC. The results correspond with many studies, such as Alzubaidi *et al*, 2015 which indicate that fluoxetine brain distribution might improve by co-administration of *Ginko Biloba*, a known inhibitor of P-gp in rats.[11] In addition, the findings of O'Brien *et al.* 2015 reported that increasing the distribution of escitalopram to the brain by P-gp inhibition with verapamil results in increased antidepressant-like activity, indicating that using a P-gp inhibitor as an additive to escitalopram therapy in these patients could be beneficial.[29]acute administration of P-gp inhibitors, such as verapamil and cyclosporin A (CsA).

CONCLUSIONS

In summary, our study shows a decrease in immobility time in FST when using 50 mg/kg, 100 mg/kg, and 200 mg/kg of *W. somnifera* for 21 days due to the antidepressant effect of the plant and due to increasing paroxetine brain concentration.

Also, RT-PCR shows a decrease in the mean fold changes in the groups that receive 50, 100, and 200 mg/kg of *W. somnifera*, indicating that *W.somnifera* has a P-gp inhibitory effect with a maximum inhibition at 100 mg/kg.

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