CHAPTER IV

RESULTS

Effect of AR root extract on NOR test

Learning and memory of animals were investigated after 90 days of administrations using NOR test. The results were expressed as percentage index of preference and recognition index. Statistical analysis revealed that there was not significantly different in the preference index among experimental groups (P=0.808) (Figure 11 (A)) while the recognition index in OVX group was significantly decreased compare to the sham group (P<0.001). However, the OVX received AR root extract 100 and 1000 mg/kg including EE 0.1 mg/kg showed significantly increased the recognition index compared to OVX group (P<0.001, P=0.026 and P<0.001, respectively) (Figure 11 (B)).

Effect of AR root extract on serum estradiol

The effects of AR on estradiol in serum were determined by ECLIA. The results were expressed as mean ± SEM (Figure 12). The serum estradiol concentration was significantly decreased in OVX group (13.13 ± 1.95 pg/ml) compared to sham group (22.91 ± 5.63 pg/ml, P=0.035). However, the significant difference of the serum estradiol were not observed among OVX, OVX+AR100 (12.71 ± 1.97 pg/ml, P=0.924), OVX+AR1000 (13.77 ± 1.51 pg/ml, P=0.887) and OVX+EE (16.09 ± 2.55 pg/ml, P=0.507).

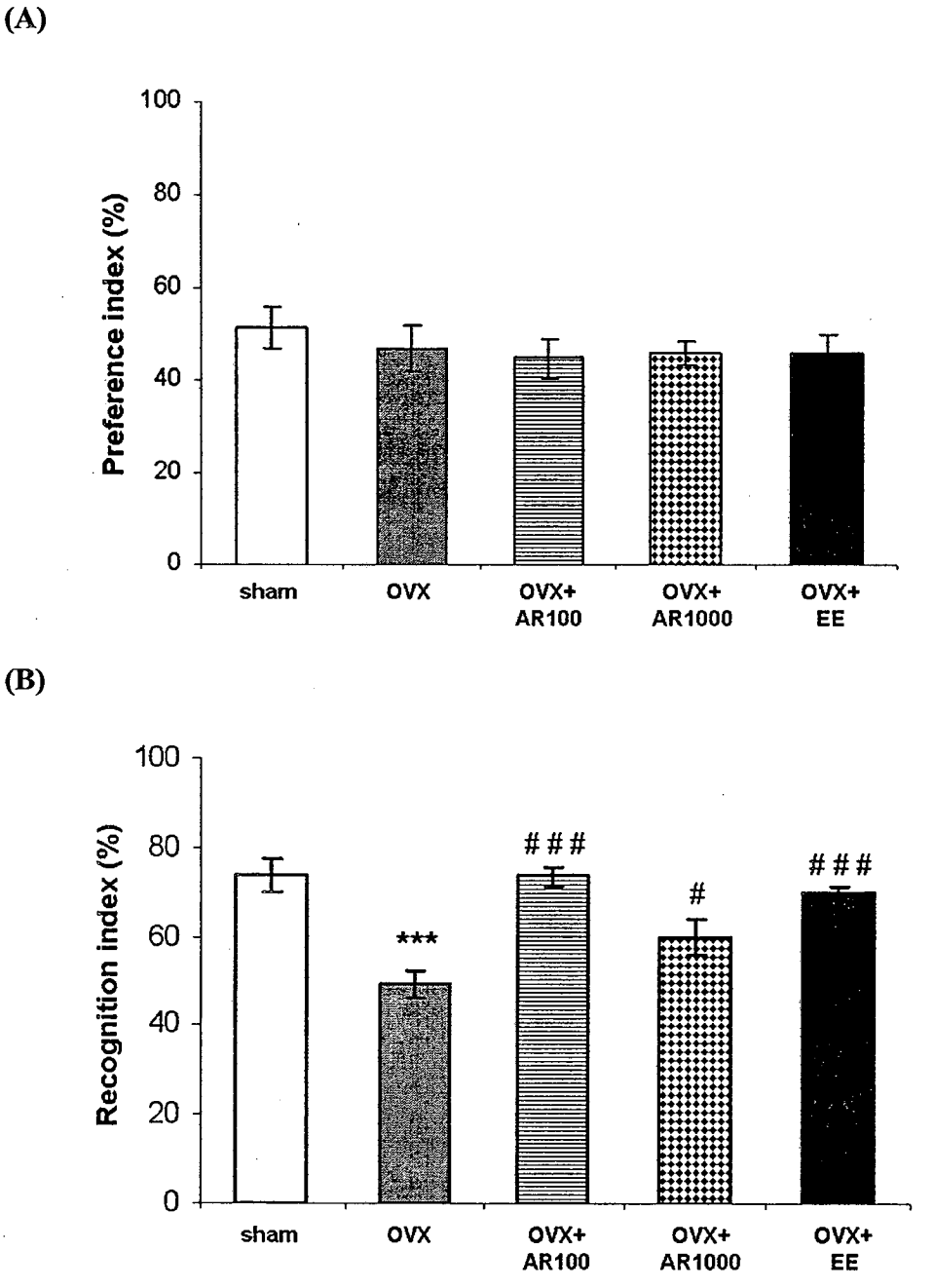


Figure 11 Effects of the AR root extract and EE on recognition memory of OVX rats were assessed by NOR test after 90 days of administrations. The data are expressed as percentage of preference index (A) and recognition index (B). Each histogram bar is expressed as mean ± S.E.M. $^{***}P < 0.001$ compared to sham group, $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared to OVX (one-way ANOVA with LSD post-hoc test).

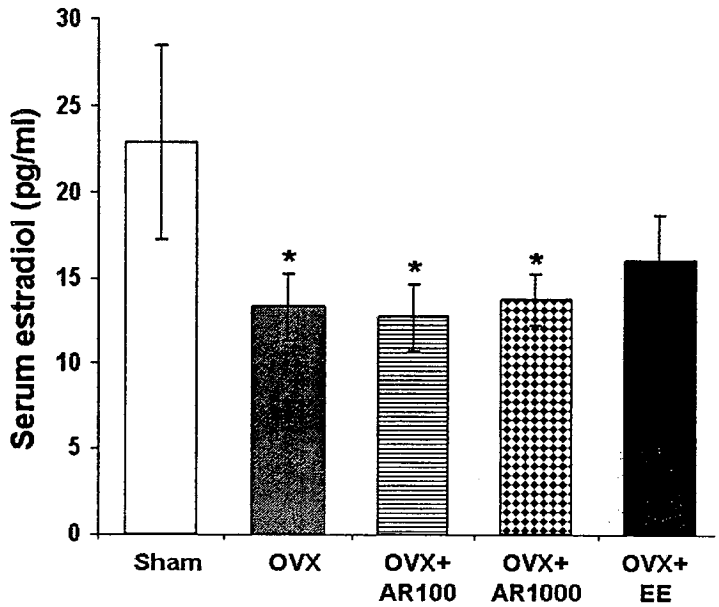


Figure 12 Effects of the AR root extract and EE on serum estradiol concentrations were assessed by ECLIA system after 90 days of administrations. Each histogram bar is expressed as mean ± S.E.M. *$P < 0.05$ compared to sham (one-way ANOVA with LSD post-hoc test).

Effect of the AR root extract on ER subtypes and BDNF protein expression

To investigate the effects of AR root extract on ER subtypes and BDNF protein expression, brain hippocampus and frontal cortex were determined by western blot analysis. Protein expression was determined by normalized the band intensity with β-actin. The control level of protein expression was considered as 100 % and the treated levels were calculated as relative percentages for each experiment.

Effect of the AR root extract on ER subtypes protein expression

The expression of hippocampal ERα protein in the OVX group was significantly reduced (36.84 ± 6.53%) when compared to the sham group (99.98 ± 25.86%, P=0.029) (Table 1 and Figure 13). The data were revealed that hippocampal ERα protein in the OVX+AR1000 and OVX+EE groups were 94.91 ± 22.65% and 131 ± 22.18%, respectively. In comparison with OVX, administration of AR 1000 mg/kg B.W. (P=0.043) and EE 0.1 mg/kg B.W. (P=0.002) were significantly increased the hippocampal expression of ERα protein. The level of ERα protein was higher in the hippocampal tissue of OVX+AR 100 (85.20 ± 9.47%), although not significant, than that OVX (P=0.087).

In the frontal cortex, ERα protein expression in OVX (36.70 ± 12.38%) was significantly decreased when compared with sham group (100.00 ± 9.17%, P=0.007). The ERα protein in OVX+AR1000 (100.22 ± 26.49%) was significantly higher than that in OVX group (P=0.006) although a significant increasing in the ERα protein was not detected in OVX+AR100 (48.91± 6.44%, P=0.566) and OVX+EE (72.55 ± 10.52%, P=0.102) compared to OVX group (Table 1 and Figure 14).

For the quantitative analysis of ERβ protein in the hippocampus, the data showed a significant differences among experimental groups (P=0.006). Post hoc analysis revealed that ERβ protein expression in OVX (48.54 ± 8.66%) was significantly lower than in sham (100.00 ± 9.88%, P=0.001) while the protein expression obtained form OVX+AR100 (100.20 ± 9.06%, P=001), OVX+AR1000 (83.66 ± 9.50%, P=0.019) as well as OVX+EE (95.17 ± 11.26%, P=0.003) were significantly increased in the ERβ protein compared with those in OVX group (Table 2 and Figure 15).

The quantitative analysis of ERβ protein expression in the frontal cortex, was also shown a significant difference among experimental groups (P=0.008). The

data revealed that the ERβ protein level in OVX was significantly reduced to 72.50 ± 4.51% compared to sham group (99.98 ± 7.85, P=0.002). However, the expression of ERβ protein in OVX+AR100 (101.38 ± 5.99%, P=0.001), OVX+AR1000 (90.93 ± 3.92%, P=0.025) as well as OVX+EE (92.51 ± 3.30%, P=0.015) were significantly increased when compared with the OVX group (Table 2 and Figure 16).

Table 1 Effect of the AR root extract on ERα protein expression

Group	Relative protein level of ERα/β-actin	
	Hippocampus	Frontal cortex
Sham	0.48 ± 0.12	0.80 ± 0.07
OVX	0.17 ± 0.03*	0.29 ± 0.10**
OVX+AR100	0.41 ± 0.04	0.39 ± 0.11
OVX+AR1000	0.43 ± 0.10$^{\#}$	0.80 ± 0.21$^{\#\#}$
OVX+EE	0.63 ± 0.10$^{\#\#}$	0.57 ± 0.08

Note: The data are represented as mean ± S.E.M. *$p < 0.05$ and **$p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$compared to OVX (one-way ANOVA with LSD post-hoc test).

Table 2 Effect of the AR root extract on ERβ protein expression

Group	Relative protein level of ERβ/β-actin	
	Hippocampus	Frontal cortex
Sham	1.79 ± 0.17	2.33 ± 0.18
OVX	0.87 ± 0.15**	1.69 ± 0.10*
OVX+AR100	1.79 ± 0.16$^{\#\#}$	2.36 ± 0.13$^{\#}$
OVX+AR1000	1.50 ± 0.17$^{\#}$	2.12 ± 0.09$^{\#}$
OVX+EE	1.70 ± 0.20$^{\#\#}$	2.16 ± 0.07$^{\#}$

Note: The data are represented as mean ± S.E.M. *$p < 0.05$ and **$p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$compared to OVX (one-way ANOVA with LSD post-hoc test).

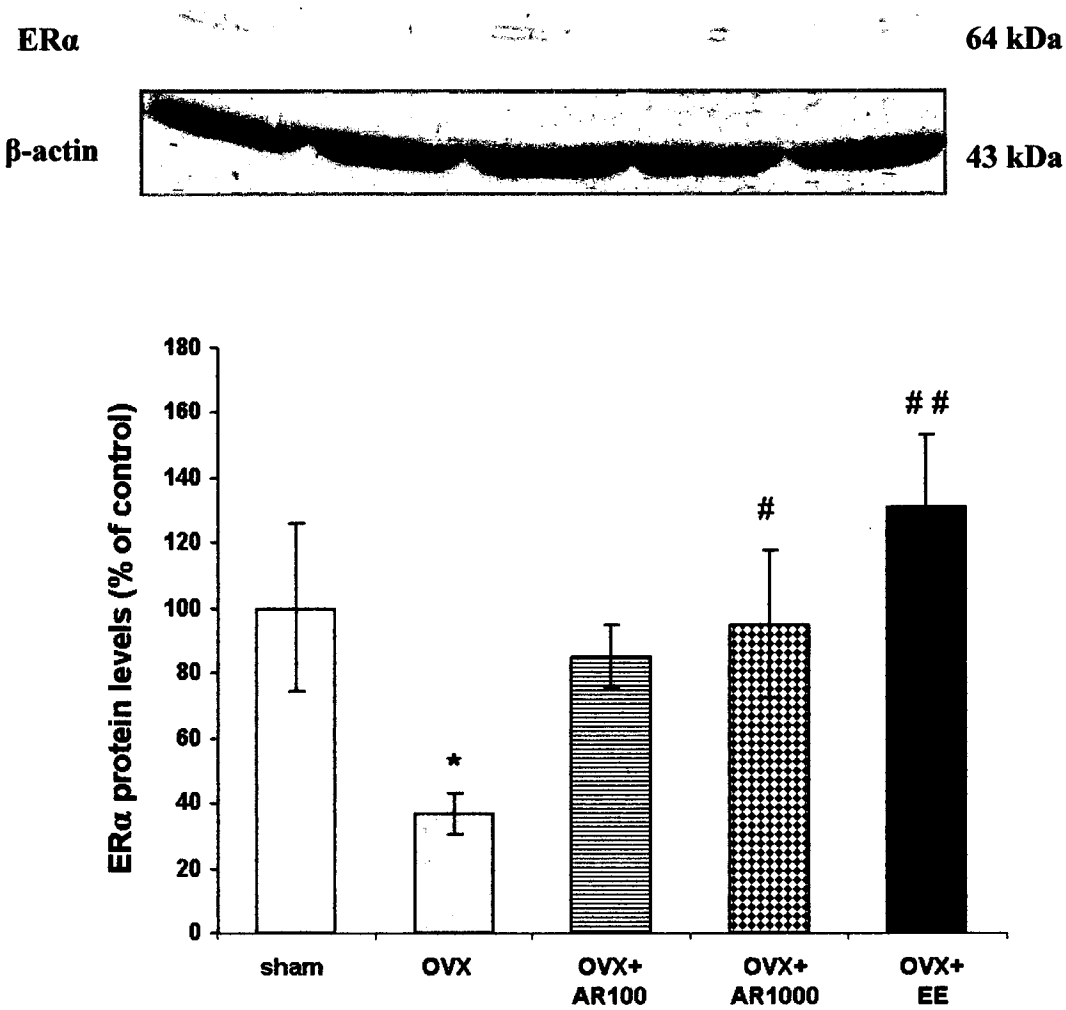


Figure 13 Effect of the AR root extract on ERα protein levels in the hippocampus were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. *$p < 0.05$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).

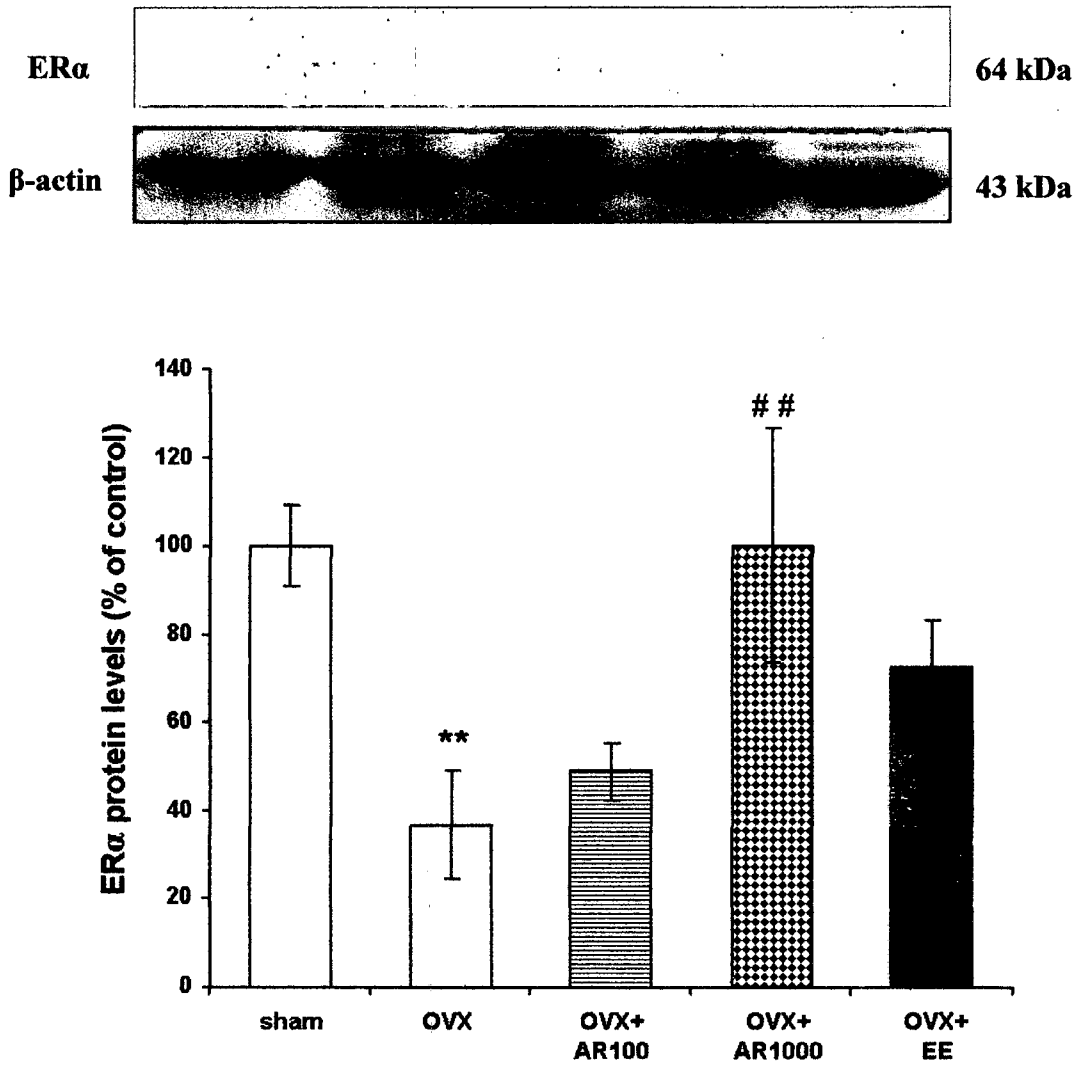


Figure 14 Effect of the AR root extract on ERα protein levels in the frontal cortex were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. **$p < 0.01$ compared to sham, $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).

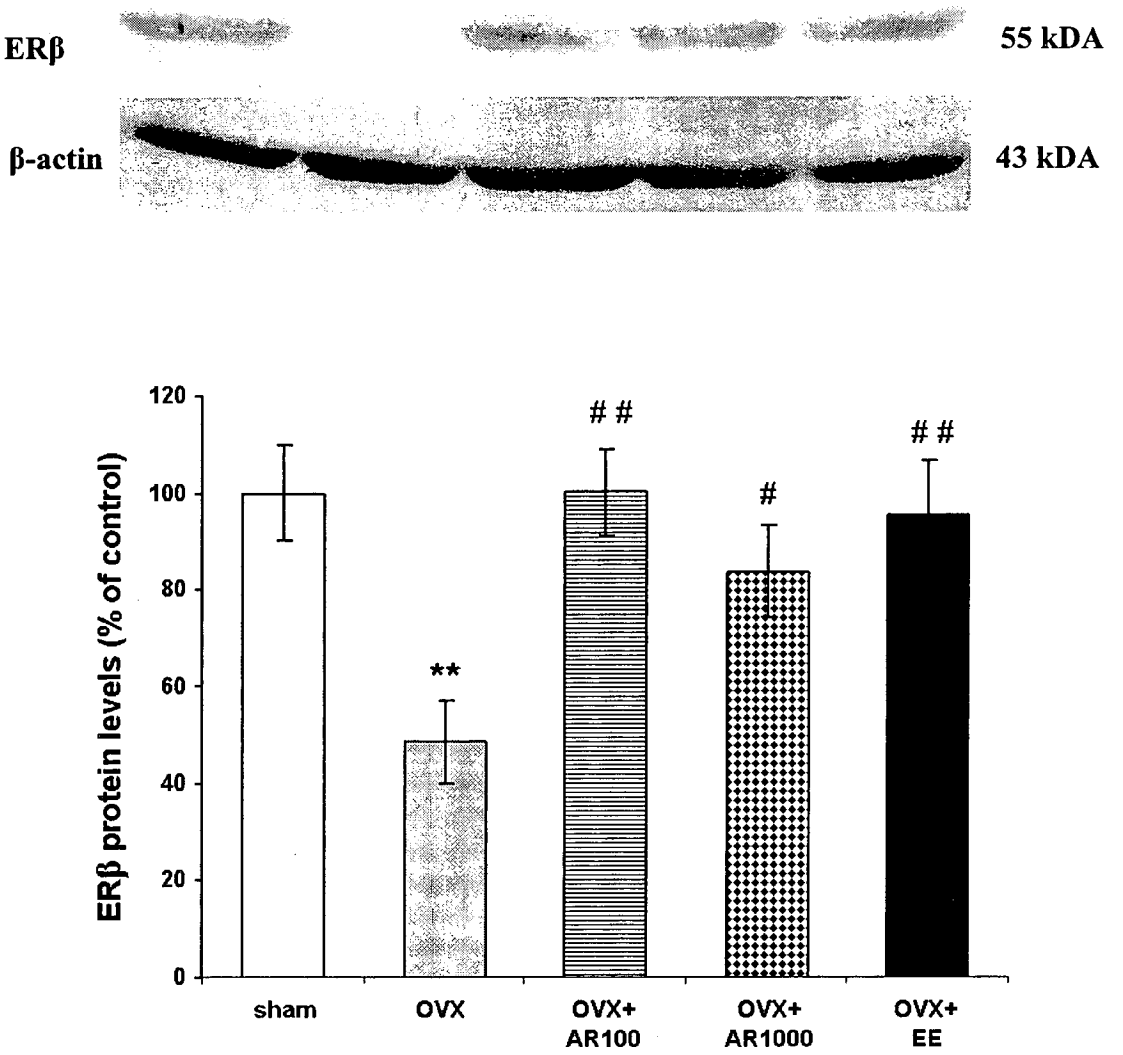
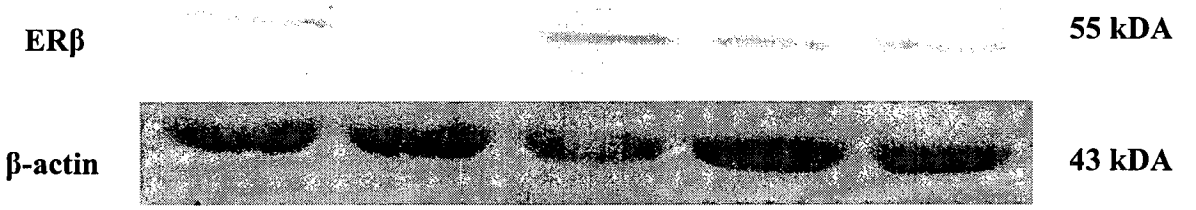


Figure 15 Effect of the AR root extract on ERβ protein levels in the hippocampus were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. **$p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).



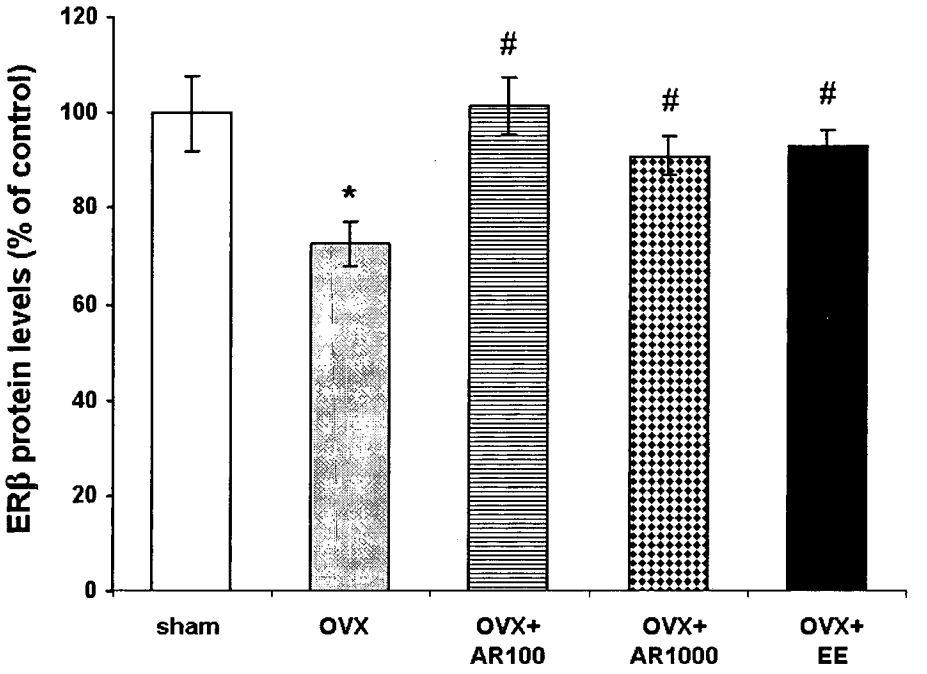


Figure 16 Effect of the AR root extract on ERβ protein levels in the frontal cortex were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. *$p < 0.05$ compared to sham, $^{\#}p < 0.05$ compared to OVX (one-way ANOVA with LSD post-hoc test).

Effect of the AR root extract on BDNF protein expression

In the hippocampus, the results demonstrated that BDNF protein level in the OVX was significantly reduced to 23.17 ± 8.94% compared with the sham group (100.00 ± 20.40%, P=0.001). However, the BDNF protein level in OVX+AR100 (84.65 ± 12.16%, P=0.005), OVX+AR1000 (67.54 ± 16.06%, P= 0.040) OVX+EE (71.40 ± 8.78%, P=0.036) were significantly increased when compared with those in the OVX group (Table 3 and Figure 17).

For the expression of BDNF protein in the frontal cortex, the statistical analysis showed that there were significant differences among experimental groups (P=0.009) (Table 3 and Figure 18). The data were revealed that BDNF protein was significantly decreased in the OVX to 38.51 ± 7.99% compared with the sham (99.99 ± 5.53%, P=0.001). The levels of BDNF protein in OVX+AR100, OVX+AR1000 and OVX+EE were 53.61 + 7.4%, 70.76 + 6.59% and 86.02 + 21.74%, respectively. The comparison analysis showed that a significant increasing in the BDNF protein levels was observed in the OVX+EE (P=0.009), although there was no significant differences in OVX+AR100 (P=0.366) and OVX+ AR1000 (P=0.062) compared to those in the OVX group.

Table 3 Effects of the AR root extract on BDNF protein expression

Group	Relative protein level of BDNF/β-actin	
	Hippocampus	Frontal cortex
Sham	0.96 ± 0.19	1.02 ± 0.05
OVX	0.22 ± 0.08**	0.39 ± 0.08**
OVX+AR100	0.81 ± 0.11$^{\#\#}$	0.51 ± 0.07
OVX+AR1000	0.64 ± 0.15$^{\#}$	0.68 ± 0.06
OVX+EE	0.67 ± 0.07$^{\#}$	0.88 ± 0.22$^{\#\#}$

Note: The data are represented as mean ± S.E.M. $^{**}p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$compared to OVX (one-way ANOVA with LSD post-hoc test).

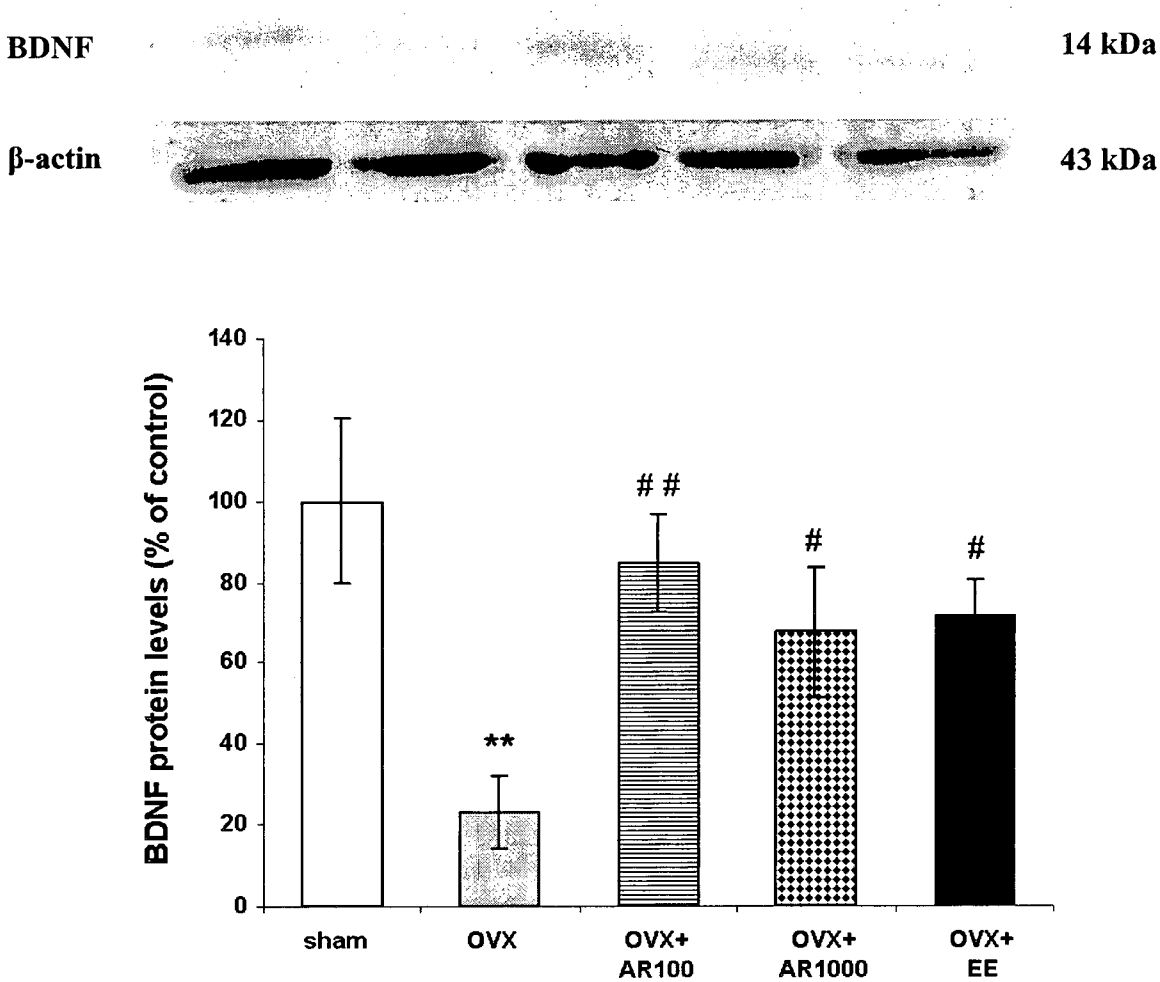
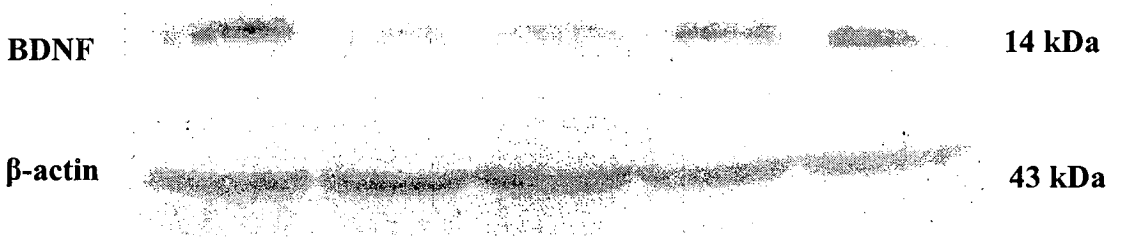


Figure 17 Effect of the AR root extract on BDNF protein levels in the hippocampus were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. $^{**}p < 0.01$ compared to sham, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).



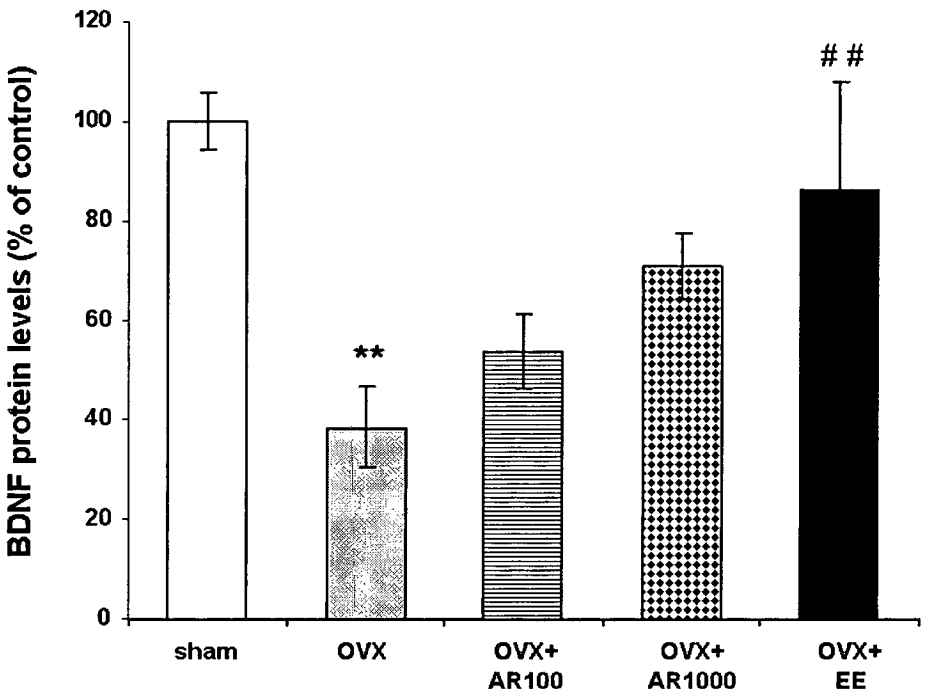


Figure 18 Effect of the AR root extract on BDNF protein levels in the frontal cortex were evaluated by western blot analysis. The data were represented as percentage value taking the sham control group as 100%. Each histogram bar is expressed as mean ± S.E.M. **$p < 0.01$ compared to sham, $^{\#\#}p < 0.01$ compared to OVX (one-way ANOVA with LSD post-hoc test).

Effect of the AR root extract on histological changes

The photomicrograph of H&E staining sections revealed the morphology of intact neurons in the CA1 (Figure 19), CA3 sub (Figure 20) and dentate gyrus sub-regions (Figure 21) of hippocampus as well as the mPFC (Figure 22). The data were expressed as mean ± SEM within an area 6,400 μm^2 of hippocampal CA1, CA3 and dentate gyrus region and 0.25 mm^2 in mPFC (Table 4).

The histological changes of neuronal cells in hippocampal CA1 region were showed no morphological lesion in sham group. Neuronal loss, shrinkage and dark staining of neurons were observed in OVX group. Number of the intact neurons in OVX (17.42 ± 0.31 cells) was significantly reduced compared with the sham (23.47 ± 2.29 cells, P=0.018). The intact neurons in OVX+AR100, OVX+AR1000 and OVX+EE were 22.78 ± 1.1, 19.18 ± 1.28 and 21.8 ± 2.83 cells, respectively. The statistical analysis revealed that administration of AR 100 mg/kg B.W. and EE 0.1 mg/kg B.W. attenuated neuronal loss caused by OVX (P=0.027 and P=0.03, respectively). There was no significant different between OVX+AR1000 and OVX groups (P=0.442).

In the hippocampal CA3 region, the results showed that number of intact neurons in the OVX group (12.6 ± 0.81 cells) was significantly decreased when compared with the sham (18.7 ± 2.02 cells, P=0.004). The intact neurons in the OVX+AR100, OVX+AR1000 and OVX+EE groups were 18.46 ± 0.7, 18.43 ± 0.23 and 17.20 ± 1.52 cells, respectively. These results established a significant increasing the number of intact neurons in the OVX+AR100 (P=0.006), OVX+AR1000 (P=0.01) as well as OVX+EE (P=0.023) when compared with OVX group.

In dendate gyrus, the intact neurons were counted within area 6,400 μm^2. The statistical analysis revealed that there was not significant differences in the number of intact neurons among experimental groups (P=0.57). The intact neurons in the sham, OVX, OVX+AR100, OVX + AR1000 and OVX + EE groups were 48.87 ± 9.28, 39.96 ± 3.56, 51.83 ± 7.04, 49.4 ± 6.21 and 40.6 ± 1.94 cells, respectively.

For evaluation the effects of the AR root extract on the number of neurons in mPFC, these cells were counted within an area 0.25 mm^2. The neurons in the OVX group (75.9 ± 15.76) was significantly decreased when compared with the sham (161.47 ± 13.24, P<0.001). The intact neurons in the OVX+AR100, OVX+AR1000

and OVX+EE were 124.43 ± 9.62, 154.8 ± 11.54 and 191.86 ± 11.92 cells, respectively. The number of intact neurons in OVX+AR100 ($P=0.014$), OVX+AR1000 ($P<0.001$) as well as OVX+EE ($P<0.001$) was significant higher than OVX.

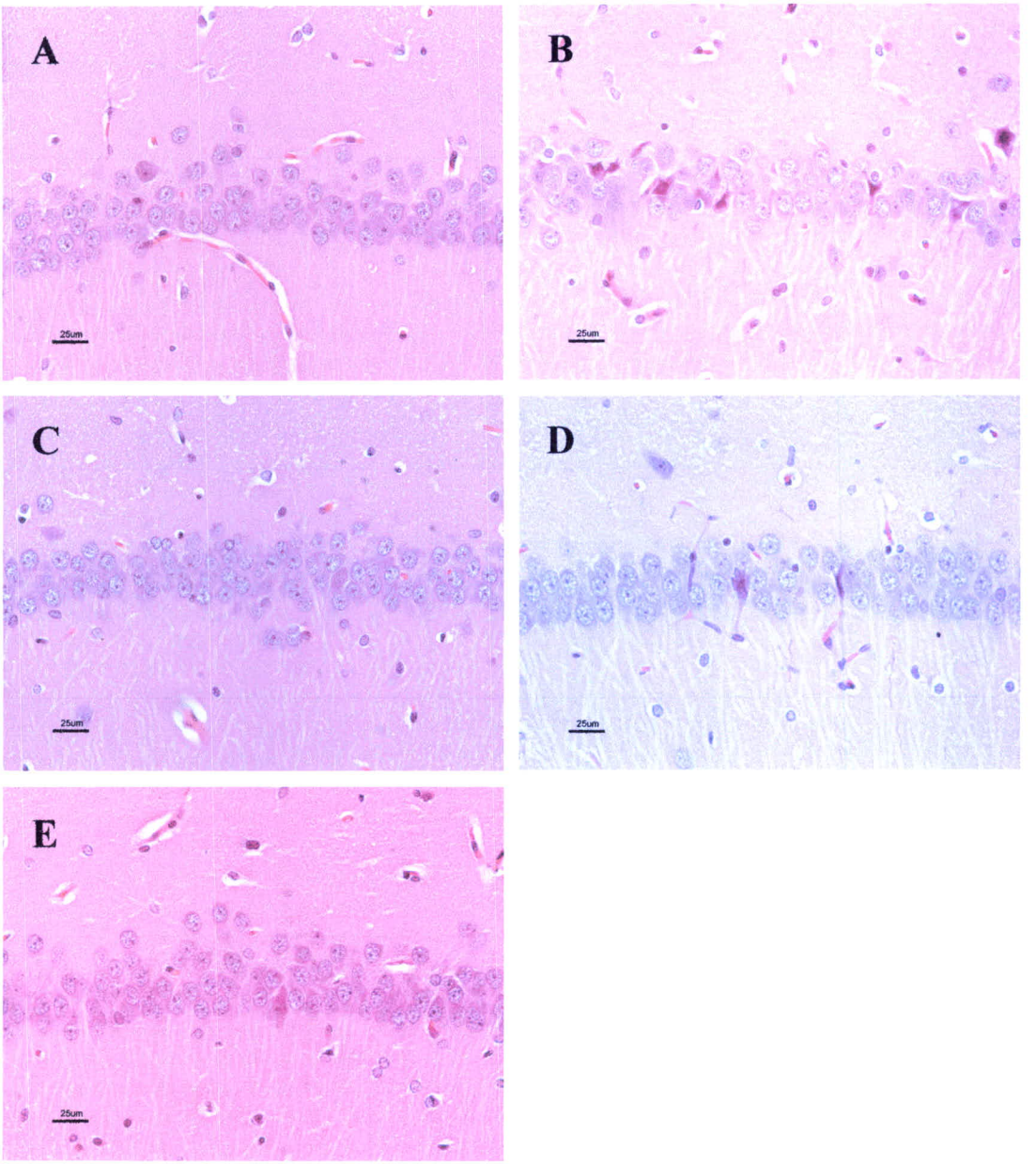


Figure 19 Microscopic photographs of neuronal densities in the hippocampal CA1 sub-region (40X)

Note: Scale bar = 25 μm, A = sham group, B = OVX group, C = OVX+AR100 group, D = OVX+AR1000 group, E = OVX+EE

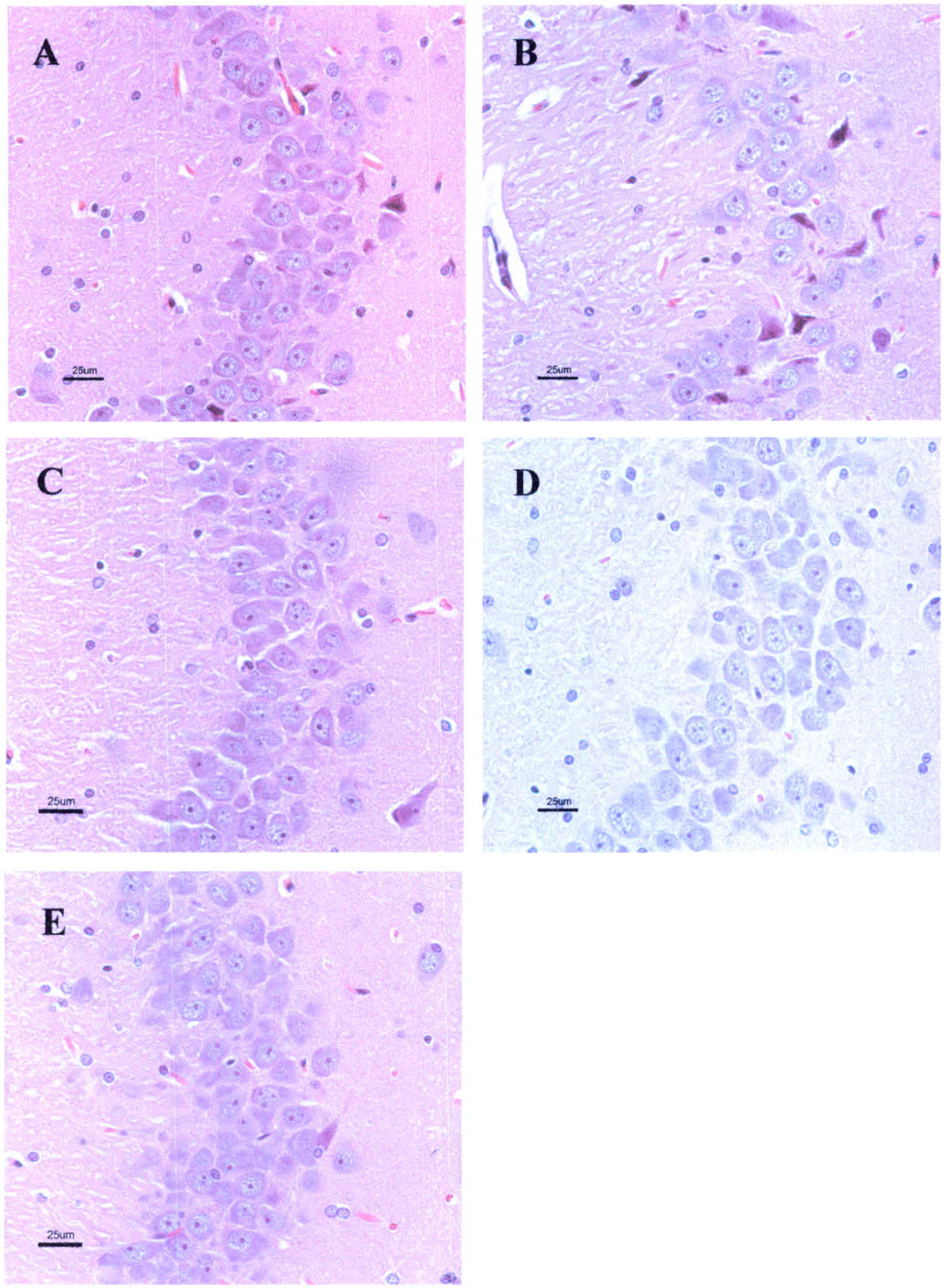


Figure 20 Microscopic photographs of neuronal densities in the hippocampal CA3 sub-region (40X)

Note: Scale bar = 25 μm, A = sham group, B = OVX group, C = OVX+AR100 group, D = OVX+AR1000 group, E = OVX+EE

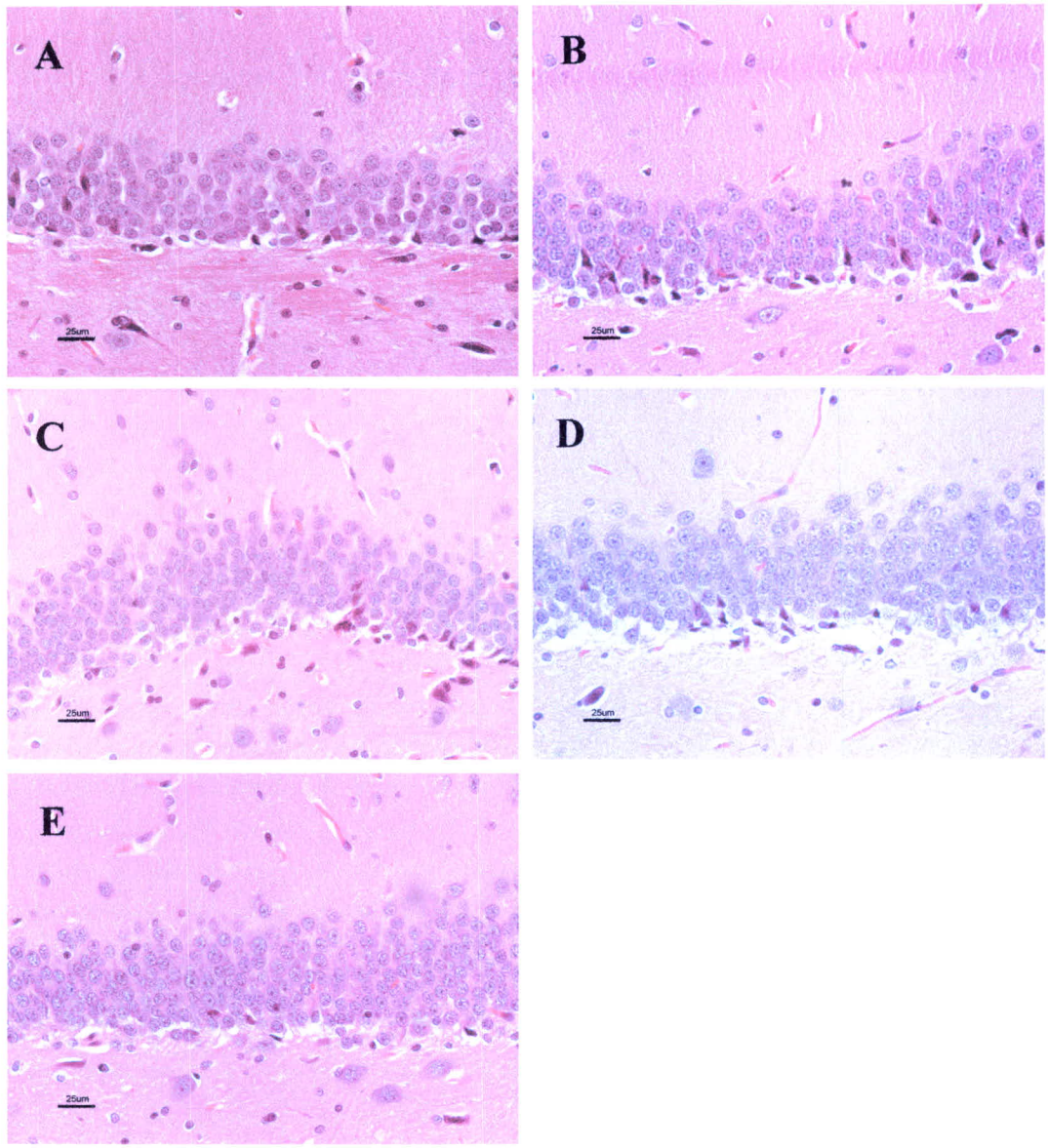


Figure 21 Microscopic photographs of neuronal densities in the dentate gyrus sub-region of hippocampus (40X)

Note: Scale bar = 25 μm, A = sham group, B = OVX group, C = OVX+AR100 group, D = OVX+AR1000 group, E = OVX+EE

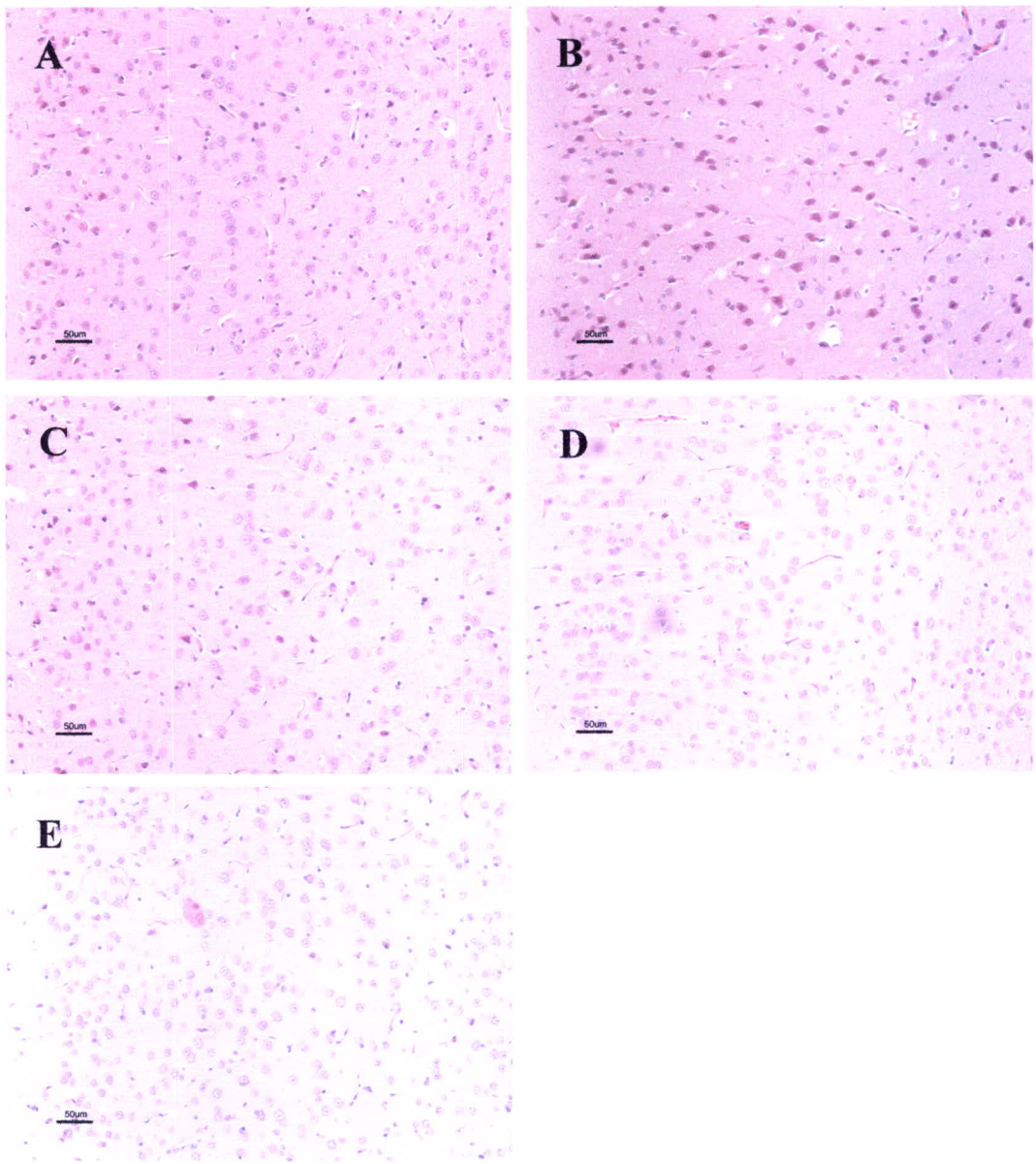


Figure 22 Microscopic photographs of neuronal densities in the medial prefrontal cortex (mPFC) area (20X)

Note: Scale bar = 50 μm, A = sham group, B = OVX group, C = OVX+AR100 group, D = OVX+AR1000 group, E = OVX+EE

Table 4 Effects of the AR root extract on intact neuronal densities

Group	Intact neuronal densities (cells/unit area)			
	CA1	CA3	DG	mPFC
Sham	23.47 ± 2.29	18.70 ± 2.02	48 ± 9.28	161.47 ± 3.24
OVX	17.42 ± 0.31*	12.60 ± 0.81**	39.96 ± 3.56	75.90 ± 15.76***
OVX+AR100	22.78 ± 1.10$^{\#}$	18.46 ± 0.70$^{\#\#}$	51.83 ± 7.04	124.43 ± 9.62$^{\#}$
OVX+AR1000	19.18 ± 1.20	18.43 ± 0.23$^{\#}$	49.40 ± 6.21	154.80 ± 11.54$^{\#\#\#}$
OVX+EE	21.80 ± 2.83$^{\#}$	17.20 ± 1.52$^{\#}$	40.60 ± 1.94	191.86 ± 11.92$^{\#\#\#}$

Note: The numbers of intact neuron were counted within area 6,400 μm^2 for hippocampal CA1, CA3 and dentate gyrus (DG) and 0.25 mm^2 for mPFC. The data are represented as mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to sham, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to OVX (one-way ANOVA with LSD post-hoc test).