CHAPTER IV
RESULTS

1. Effect of *Glycosmis parva* solvent extracts on LPS stimulated-macrophages

All solvent extracts from branches and leaves of *G. pava* were identified for their inhibitory activities on LPS-stimulated J774A.1. Inhibition of nitric oxide synthesis from the stimulated cells was used to evaluate these extracts. J774A.1 cells were pretreated with 10 - 100 μg/ml of each extract for 24 h and then treated with 100 ng/ml LPS for the next 24 h. The supernatant from the treated cells was collected for determining NO content by Griess reaction. The hexane and the ethyl acetate extracts from both branches (G1 and G2) and leaves (G5 and G6) of *G. pava* clearly inhibited NO production in LPS-stimulated J774A.1 cells (Fig.9a). However, all these extracts, except the hexane extract from branches (G1), at 100 μg/ml concentration also had highly cytotoxic effect on the treated cells (Fig.9b). These extracts were employed in the subsequent studies. The butanol and the water extracts of branches (G3 and G4) and leaves (G7 and G8) didn't have both the inhibitory and the cytotoxic effects to the cells. These extracts were not studied further in the subsequent studies.

The hexane and the ethyl acetate extracts from branches and leaves of *G. pava* (G1, G2, G5, and G6, respectively) were determined for their 50% inhibitory concentrations (IC_{50}'s) on NO production from LPS-stimulated J774A.1. The cells were pretreated with G1 (6.25-100 μg/ml), G2 (3.13-50 μg/ml), G5 and G6 (1.56-50 μg/ml) for 24 h and then treated with 100 ng/ml LPS for 24 h. The supernatant from the treated cells was collected for determining NO content by Griess reaction and the cells were used to determine the cytotoxicity of the extracts by resazurin staining assay. All extracts inhibited NO production in LPS-stimulated cells in a concentration-dependent manner (Fig.10a-13c). Their IC_{50} values for the NO production were 44.70, 16.70, 11.76 and 11.19 μg/ml in G1, G2, G5 and G6, respectively. The potencies of these extract were in the following order; G5 = G6 > G2 > G1. These IC_{50} values were used for

selecting concentrations of the extracts to study their molecular activities in the following studies.

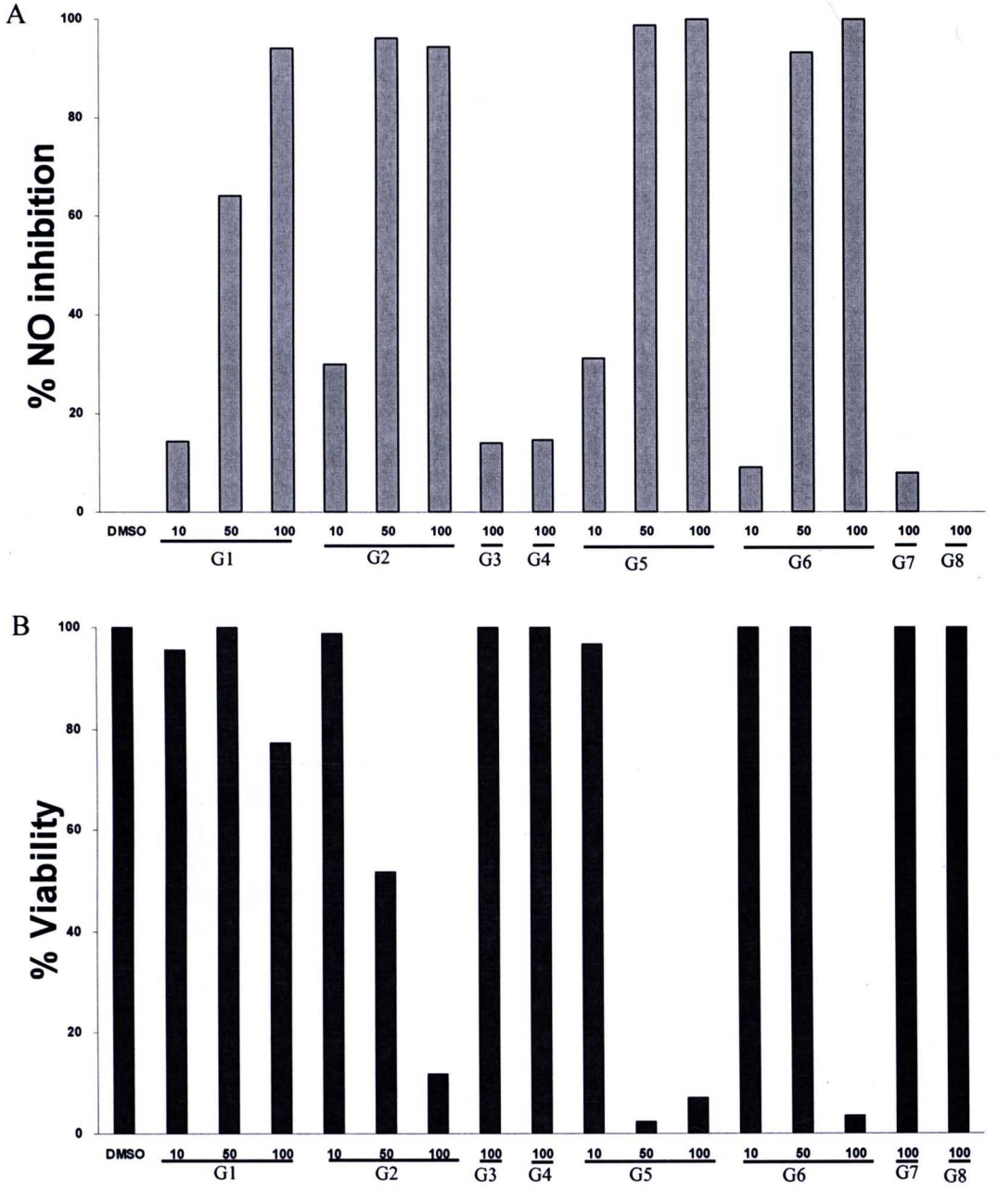


Figure 9: (A) Inhibition effect *G. parva* on NO production in LPS stimulated-macrophage J774A.1 at dose 10 -100 µg/ml of extracts. (B) Cytotoxic effect of *G. parva* in LPS stimulated-macrophage J774A.1 at dose 10 -100 µg/ml of extracts [The extracts from branches: G1: hexane, G2: ethyl acetate, G3: butanol, G4: water; The extracts from leaves: G5: hexane, G6: ethyl acetate, G7: butanol, G8: water]. Results are means ± S.D. (N=2).

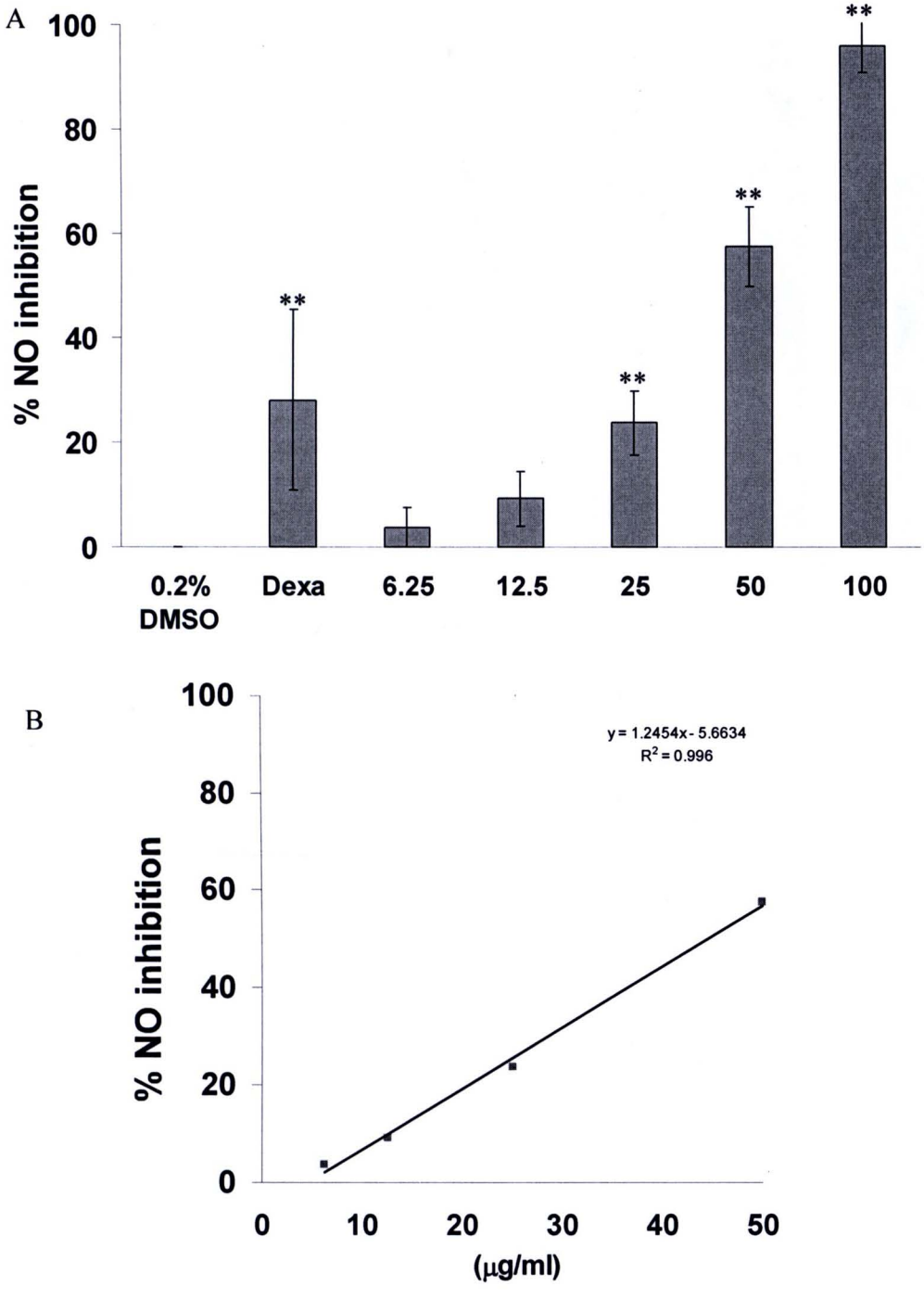


Figure 10: (A) Inhibition effect of G1 on NO production in LPS stimulated-macrophage J774A.1 at dose 6.25-100 µg/ml of extracts. (B) IC_{50} of G1 (44.69 µg/ml). ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=8).

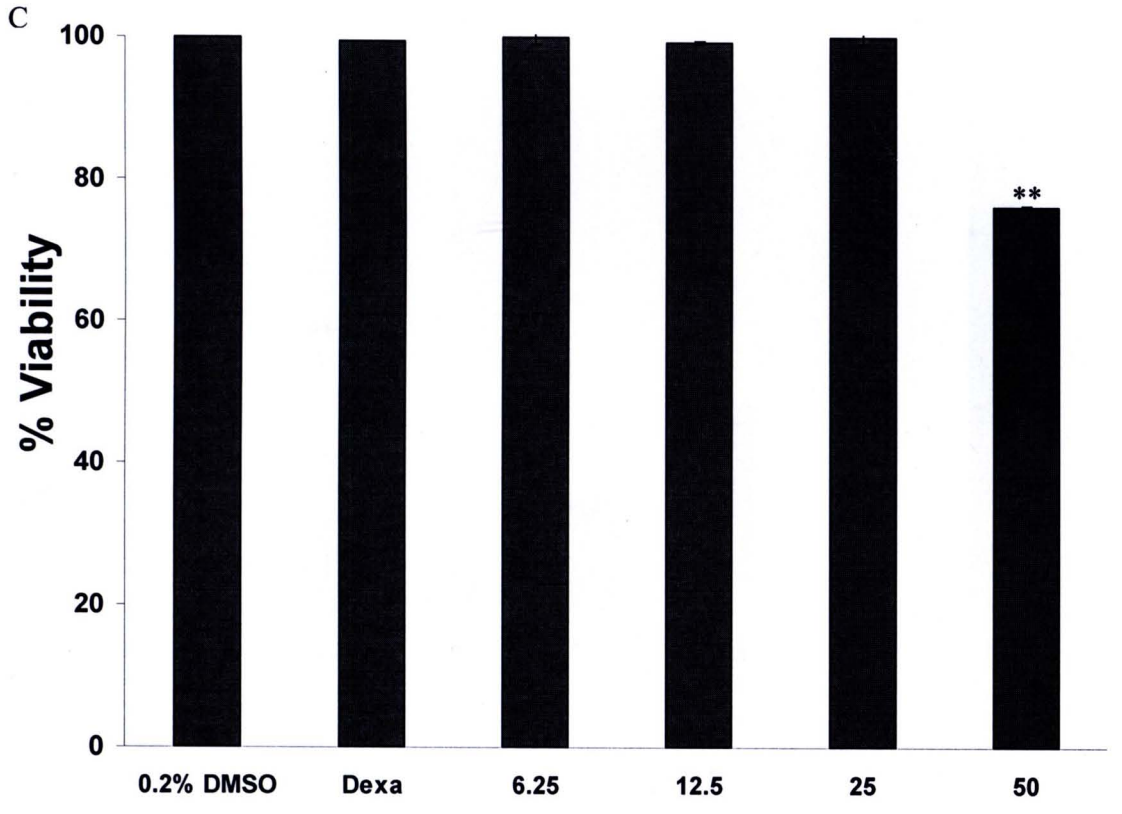


Figure 10: (C) Cytotoxic effect of G1 in LPS stimulated-macrophage J774A.1 at dose 6.25-100 µg/ml of extracts. ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=8).

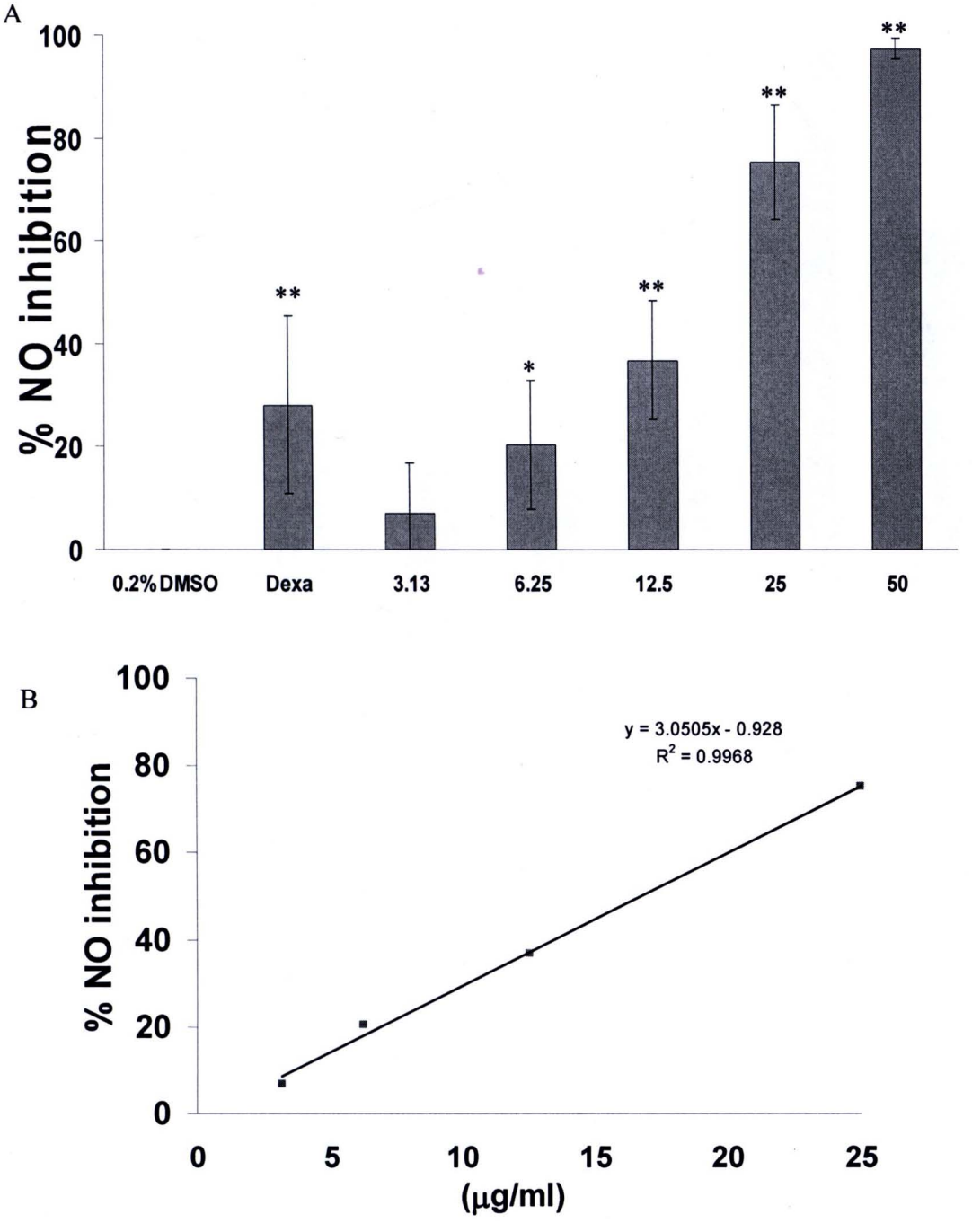


Figure 11: (A) Inhibition effect of G2 on NO production in LPS stimulated-macrophage J774A.1 at dose 3.13 - 50 µg/ml of extracts. (B) IC_{50} of G2 (16.69 µg/ml). * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=8).

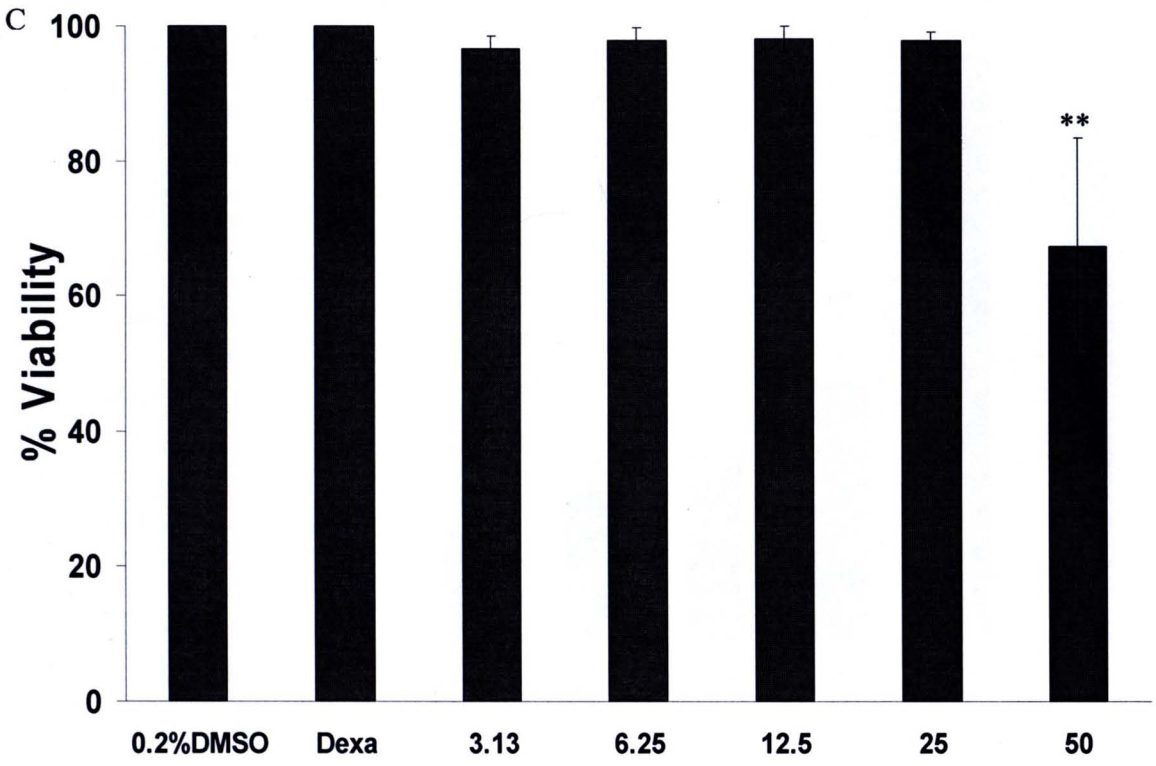


Figure 11: (C) Cytotoxic effect of G2 in LPS stimulated-macrophage J774A.1 at dose 3.13 - 50 µg/ml of extracts. ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=8).

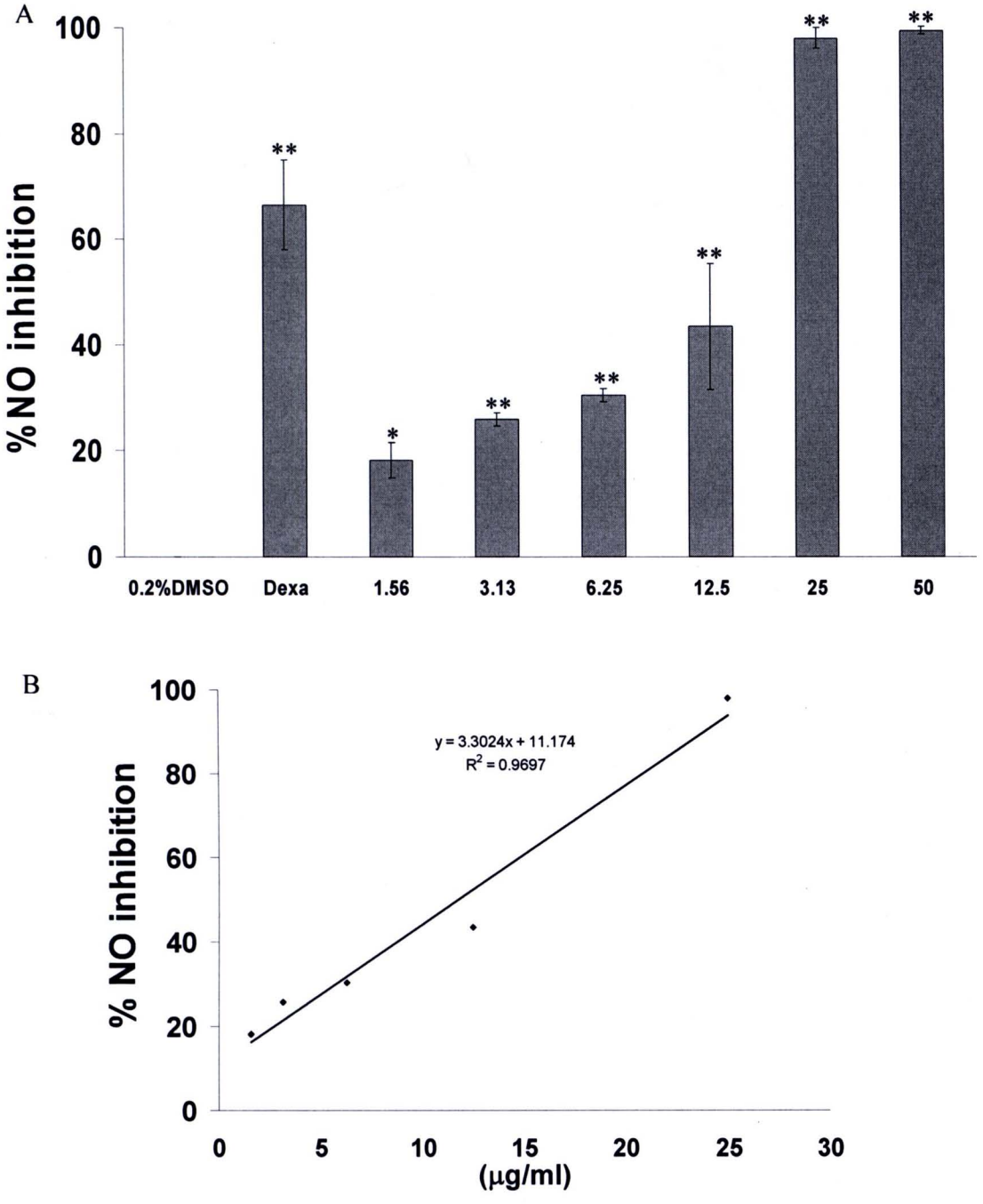


Figure 12: (A) Inhibition effect of G5 on NO production in LPS stimulated-macrophage J774A.1 at dose 1.56- 50 µg/ml of extracts. (B) IC_{50} of G5 (11.57 µg/ml). * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$) Results are means ± S.D. (N=3).

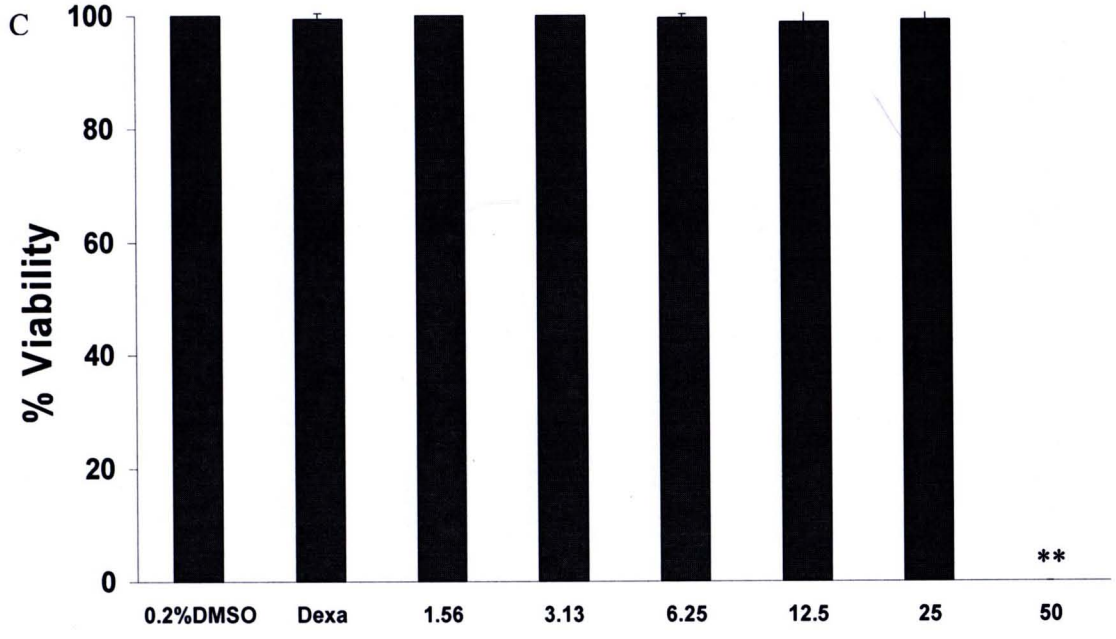


Figure 12: (C) Cytotoxic effect of G5 in LPS stimulated-macrophage J774A.1 at dose 1.56 - 50 µg/ml of extracts. * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=3).

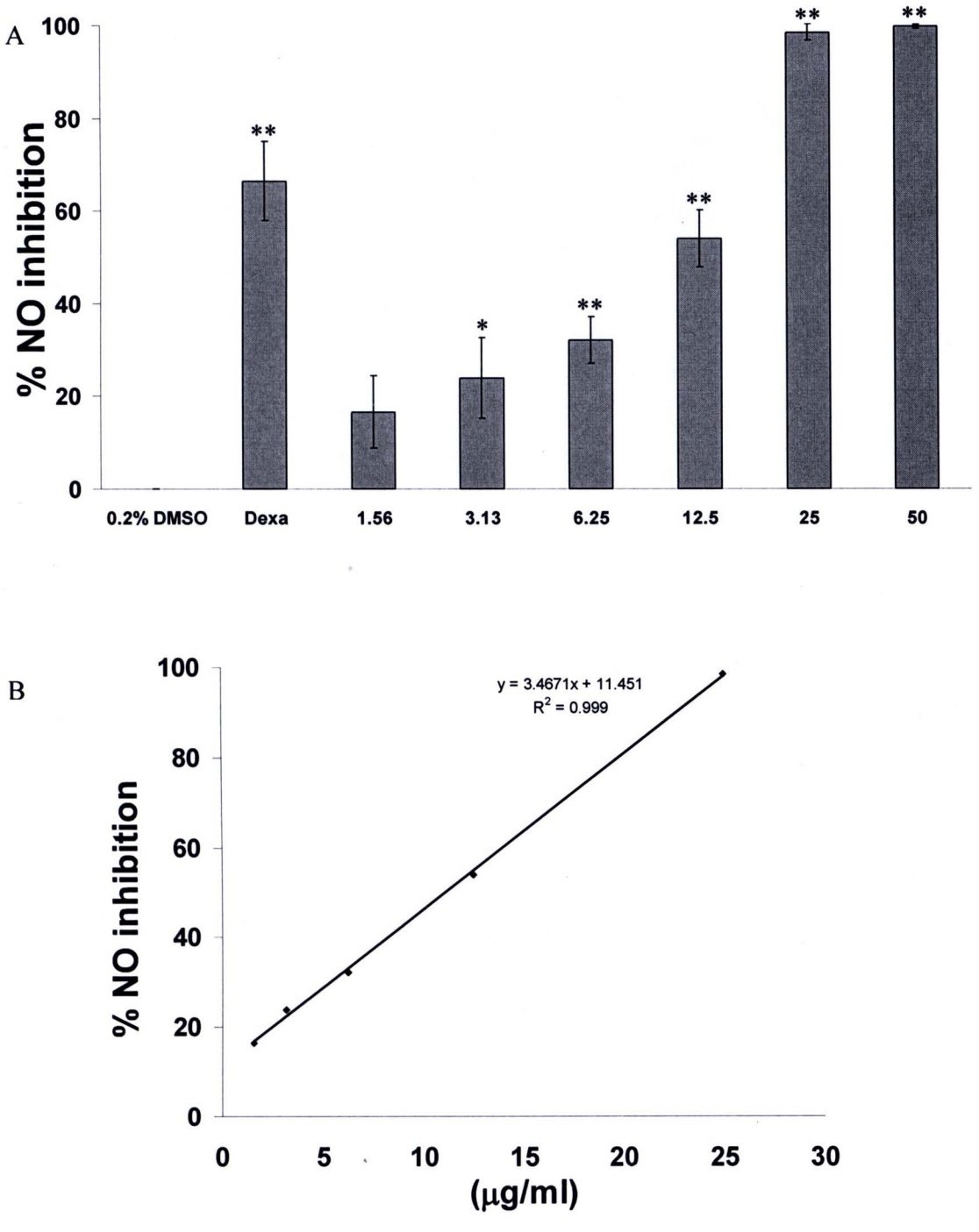


Figure 13: (A) Inhibition effect of G6 on NO production in LPS stimulated-macrophage J774A.1 at dose 1.56 - 50 µg/ml of extracts. (B) IC_{50} of G6 (11.12 µg/ml). * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=3).

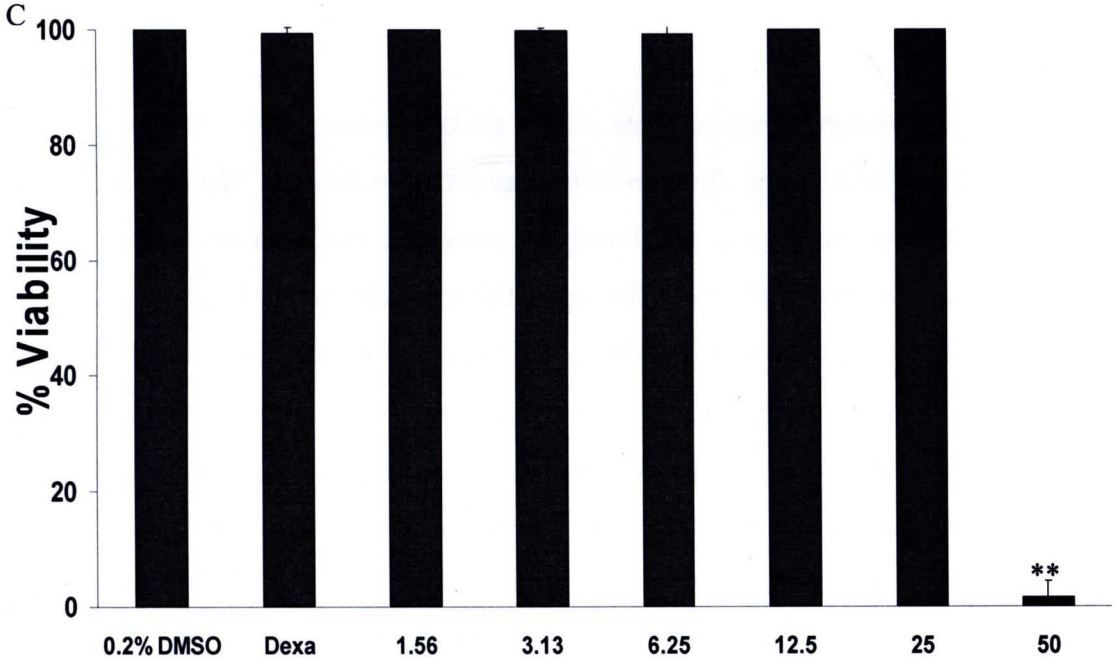


Figure 13: (C) Cytotoxic effect of G6 in LPS stimulated-macrophage J774A.1 at dose 1.56 -50 µg/ml of extracts. * significantly different between 0.2% DMSO and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO and test compounds ($p<0.001$). Results are means ± S.D. (N=3).

2. Effect of *Glycosmis parva* extracts on the expressions of pro-inflammatory cytokines in LPS stimulated-macrophages.

The effects of the hexane and the ethyl acetate extracts from branches (G1 and G2) and leaves (G5 and G6) of *G. parva* on TNF-α, IL-1β, and IL-6 in LPS-stimulated J774A.1 were evaluated. The cells were pretreated with 2- or 3 concentrations of the extracts for 24 h and then treated with 100 ng/ml LPS for 4 h. The total RNA was isolated from the treated cells and used to determine the expression of TNF-α, IL-1β, and IL-6 by RT-PCR. All extracts were used at the IC_{50} for NO inhibition from the previous study plus one or two concentrations which were 2-folds lower and higher than the IC_{50}.

G1 at the concentrations of 25 and 50 μg/ml was used in these experiments. Its IC_{50} was 44.70 μg/ml. It inhibited the expression of the pro-inflammatory cytokines, TNF-α (23.4, 54.3% respectively), IL-1β (13.0%, 15.9%), and IL-6 (9.4, 56.1%) (Appendix B-12) in LPS-stimulated J774A.1. It had inhibitory effect on the expression of TNF-α and IL-6 expression higher than on the expression of IL-1β at 50 μg/ml (Fig. 14).

Ten and 20 μg/ml G2 was used in the study. Its IC_{50} was 11.70 μg/ml. It significantly inhibited the expression of TNF-α (57.8%) and IL-1β (69.85%) (Appendix B-14) at 20 μg/ml, but it didn't have effect on the expression of IL-6 at this concentration (Fig.15).

The IC_{50} of NO inhibition of G5 was 11.76 μg/ml. It was used in this study at 6.25, 12.5 and 25 μg/ml. It profoundly inhibited the expression of TNF-α (91.1, 93.02, 94.27%) (Appendix B-16) in all concentrations used (Fig.16). The inhibition effect of G5 on IL-1β was found only at high concentration (25 μg/ml). It didn't have effect on IL-6 expression.

The IC_{50} of NO inhibition of G6 was 11.20 µg/ml. It was used in this study at 6.25, 12.5 and 25 µg/ml. The results in Fig.17 demonstrated that it significantly decreased the expression of the pro-inflammatory cytokines at its IC_{50}. At 25 µg/ml, it greatly reduced the expression of TNF-α by 30.2- 98.6 %. For IL-1β only the concentration at 25 µg/ml of G6 significantly inhibited its expression by 35.2%. While the concentration of G6 at 12.5, 25 µg/ml significantly inhibited IL-6 by 55.48 and 97.94%. (Appendix B-18) These results suggest that these solvent extracts of *G. parva* have different patterns of pro-inflammatory cytokine inhibition. They may contain different constituents.

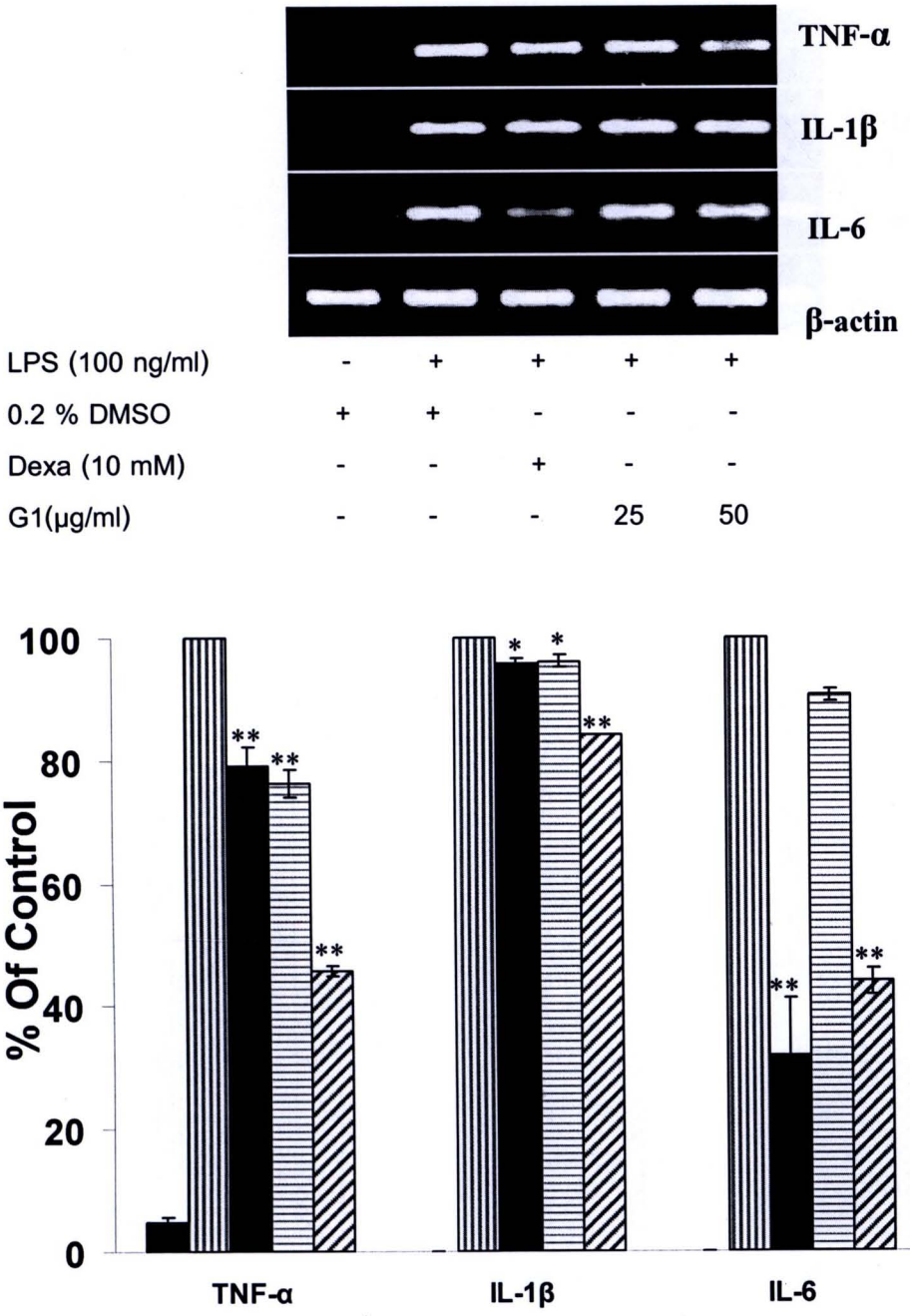
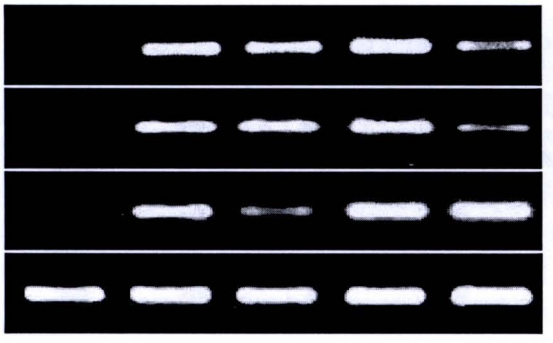


Figure 14 : Effect of G1 on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2). ■ 0.2% DMSO, ▥ 0.2%DMSO+LPS, ■ Dexamethasone 10μM+LPS , ▤ G1 25 μg/ml +LPS, ▨ G1 50 μg/ml + LPS.



LPS (100 ng/ml)	-	+	+	+	+
0.2 % DMSO	+	+	-	-	-
Dexa (10 mM)	-	-	+	-	-
G2(μg/ml)	-	-	-	10	20

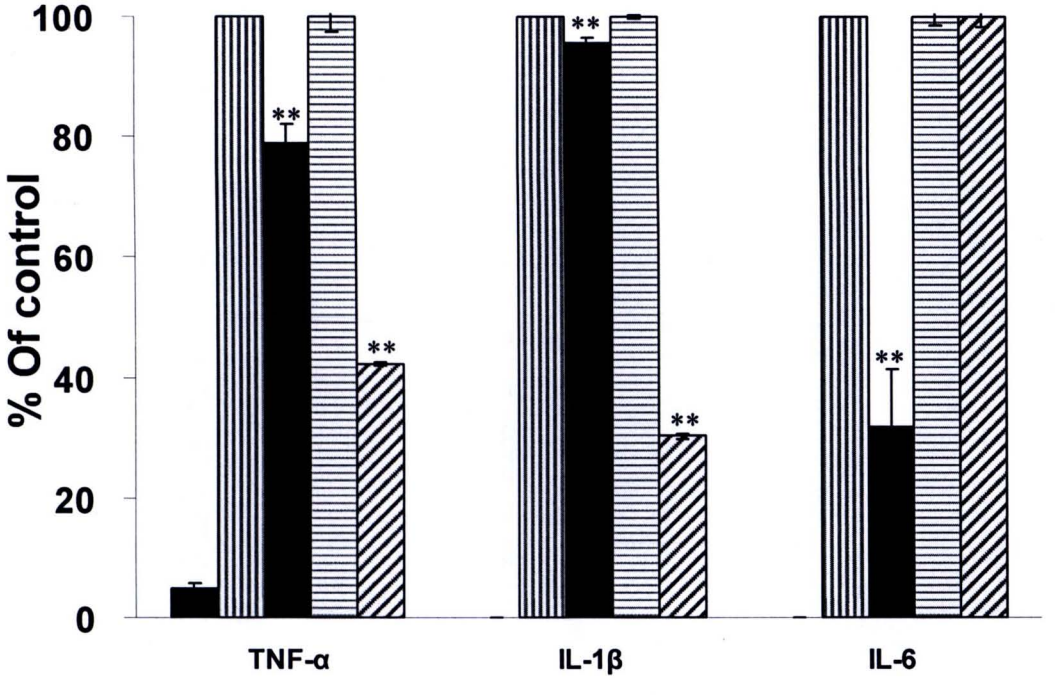
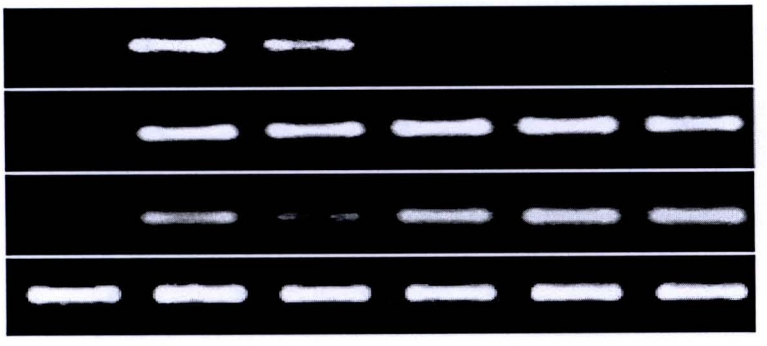


Figure 15 : Effect of G2 on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO + LPS and test compounds($p<0.001$). Results are means ± S.D. (N=2). ■ 0.2% DMSO, ▥ 0.2%DMSO+LPS, ■ Dexamethasone 10 μM+LPS, ▤ G2 10 μg/ml +LPS, ▨ G2 20 μg/ml +LPS.



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G5 (μg/ml)	-	-	-	6.25	12.5	25

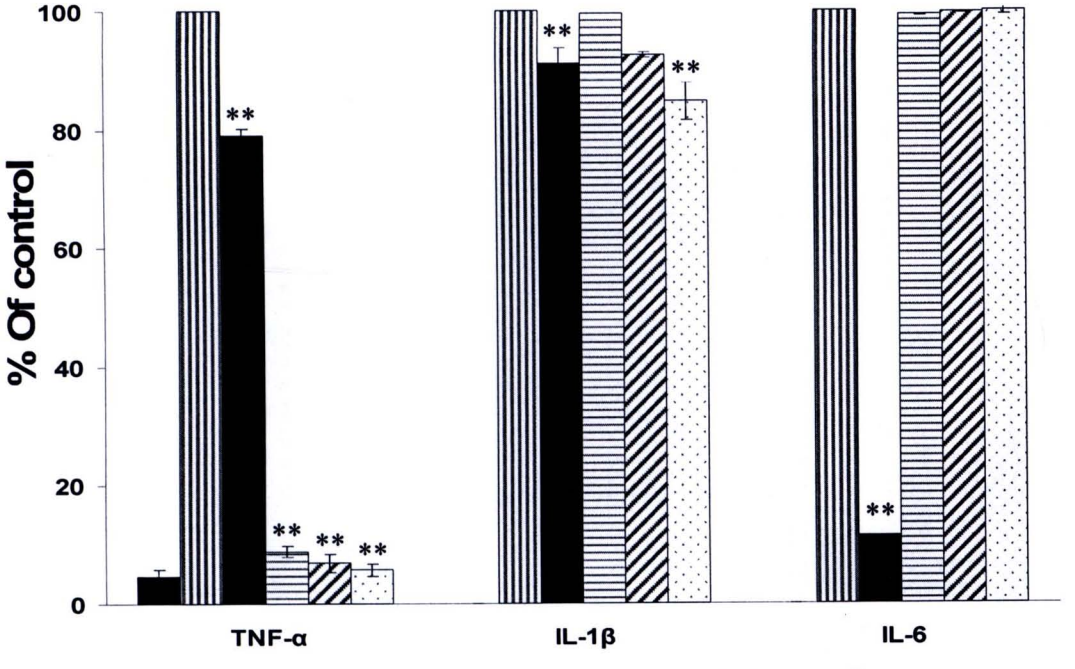
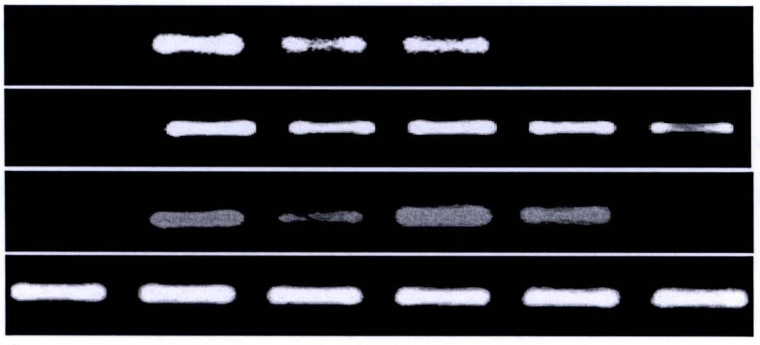


Figure 16 : Effect of G5 on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2). ■ 0.2% DMSO, ▥ 0.2%DMSO+LPS, ■ Dexamethasone 10 μM+LPS , ▤ G5 6.25 μg/ml +LPS, ▨ G5 12.5 μg/ml +LPS, ⬚ G5 25 μg/ml +LPS.



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G6 (μg/ml)	-	-	-	6.25	12.5	25

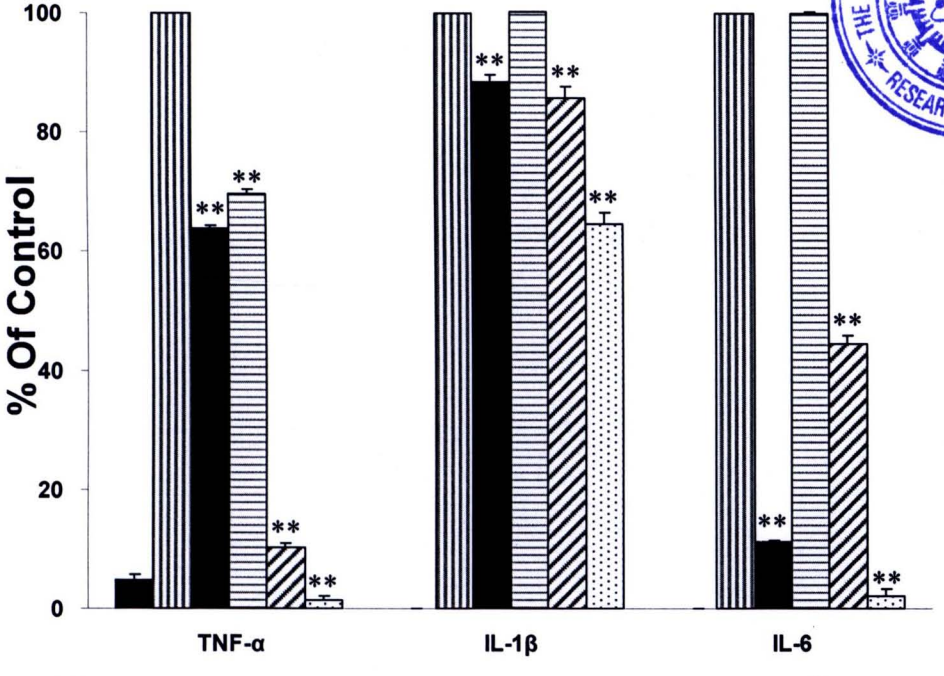
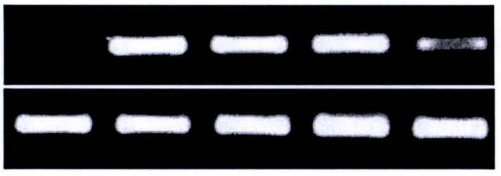


Figure 17 : Effect of G6 on mRNA expressions of cytokines (TNF-α, IL-1β and IL-6) in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2). ■ 0.2% DMSO, ▥ 0.2%DMSO +LPS, ■ Dexamethasone 10 μM+LPS, ▤ G6 6.25 μg/ml +LPS, ▨ G6 12.5 μg/ml +LPS, ⁝⁝ G6 25 μg/ml +LPS.

3. Effect of *Glycosmis prava* extracts on the expressions of iNOS in LPS stimulated-macrophages.

The inhibitory effects of G1, G2, G5 and G6 of *G. parva* on the activity of iNOS enzyme in J774A.1 were examined whether they correlated with their activity on NO production. All extracts were used at their IC_{50} for NO inhibition from the previous study plus one or two concentrations in 2-fold dilution. The cells were pretreated with the extracts for 24 h and then treated with 100 ng/ml LPS for 24 h. The total RNA was isolated from the treated cells and used to determine the expression of iNOS by RT-PCR. The results in Fig.20-21 demonstrated that the extracts inhibited iNOS expression in a concentration-dependent manner for G5 and G6. Fraction of G6 (25 µg/ml) seemed to produce most pronounced effect (82.52%) (Fig. 21, Appendix B-25 and B-30). Only high concentration of G2 (20 µg/ml) could significantly inhibit the mRNA expression of iNOS(Fig. 19, Appendix B-22 and B-28). These results correlated with the effects of these extracts on NO production.



LPS (100 ng/ml)	-	+	+	+	+
0.2 % DMSO	+	+	-	-	-
Dexa (10 mM)	-	-	+	-	-
G1(μg/ml)	-	-	-	25	50

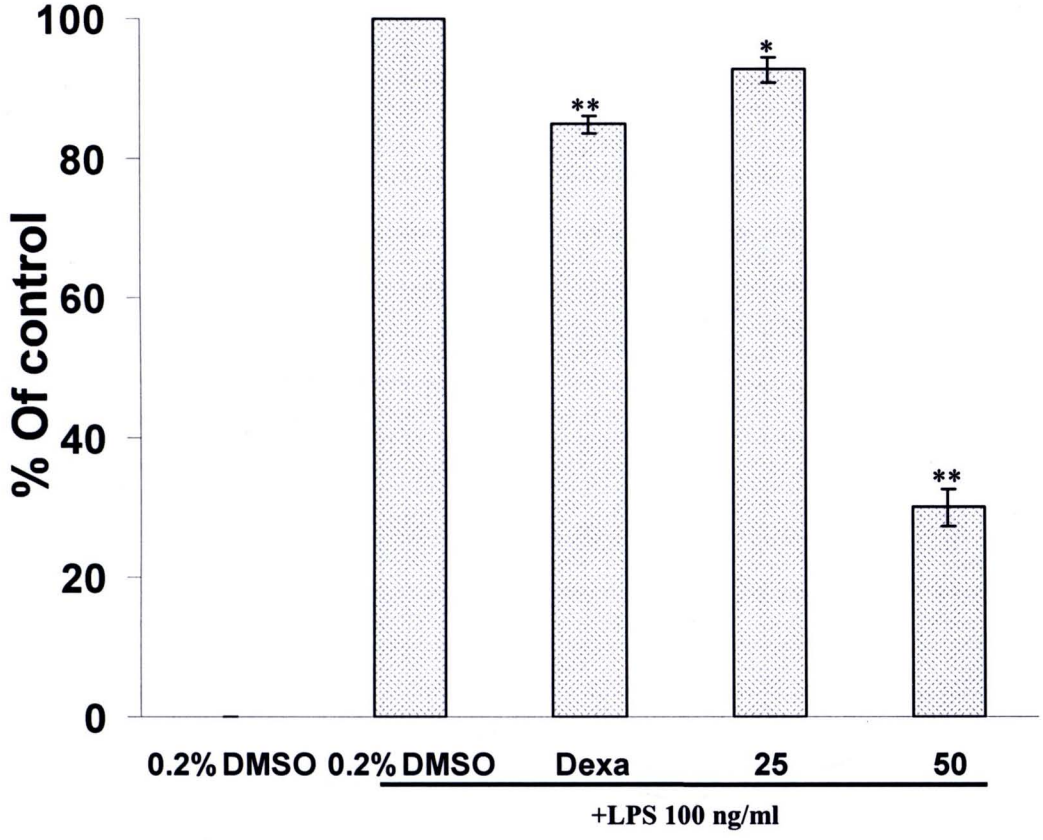
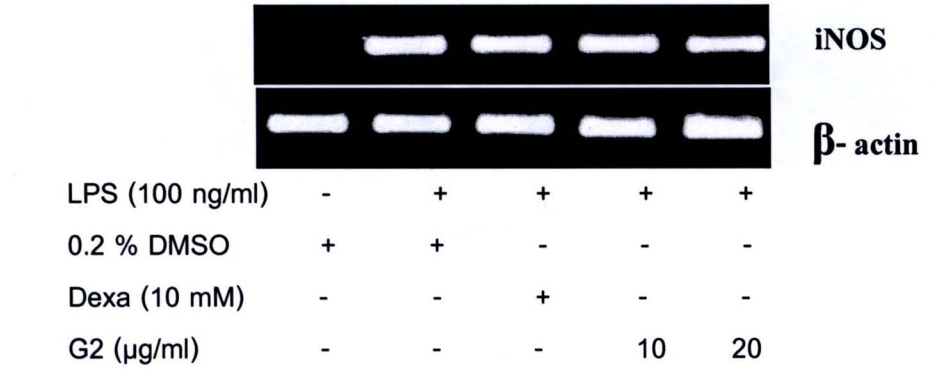


Figure 18 : Effect of G1 on mRNA expressions of iNOS in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).



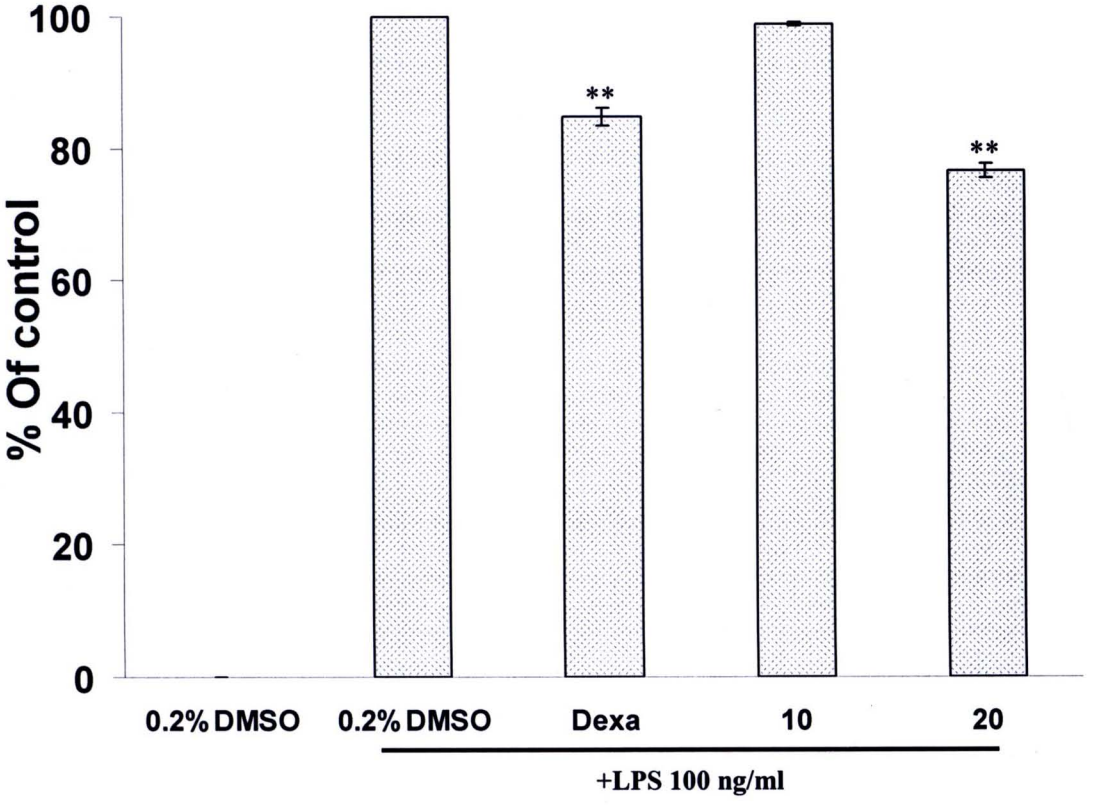
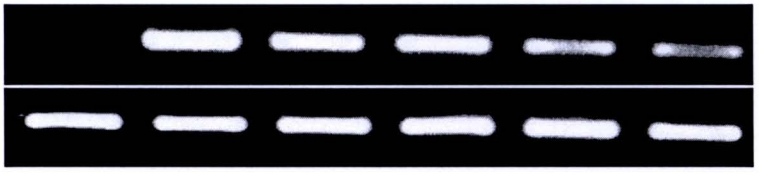


Figure 19 : Effect of G2 on mRNA expressions of iNOS in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G5 (μg/ml)	-	-	-	6.25	12.5	25

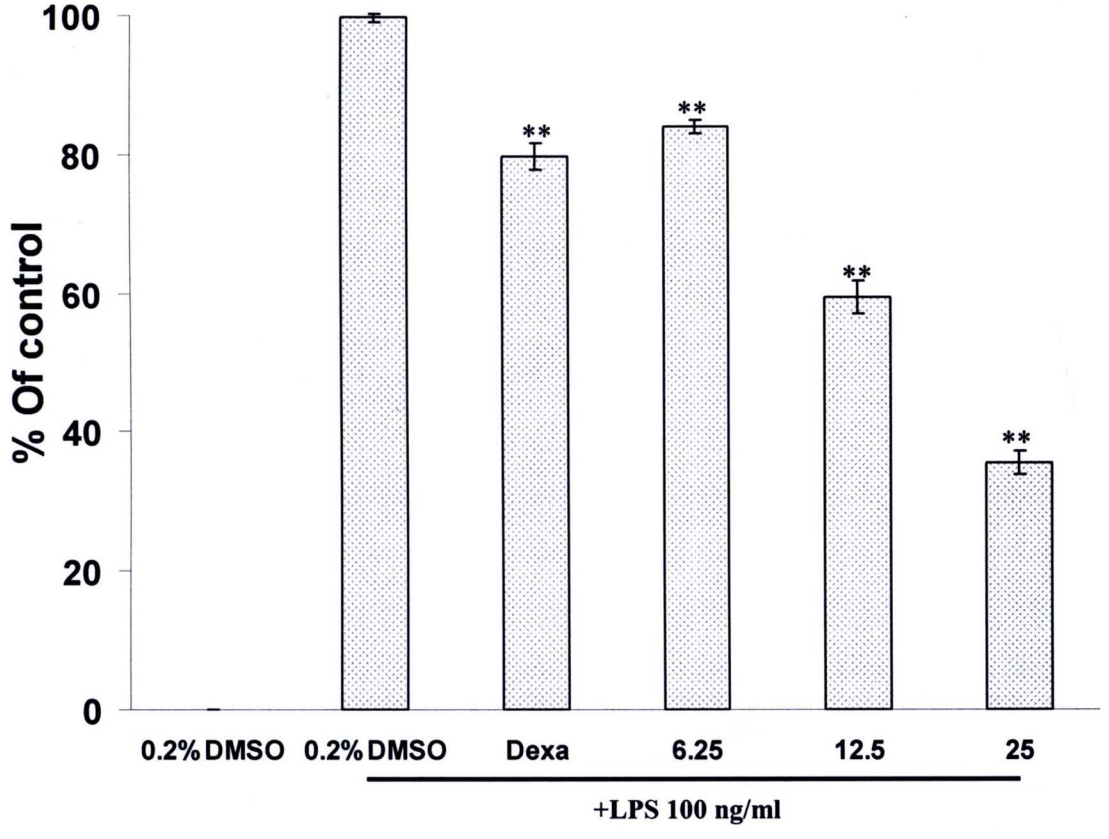
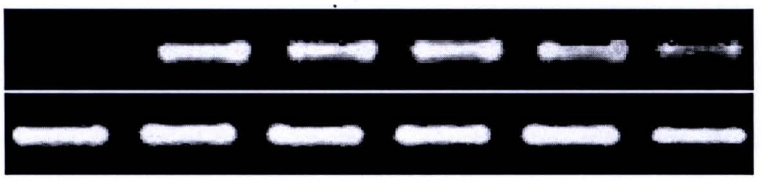


Figure 20 : Effect of G5 on mRNA expressions of iNOS in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G6 (μg/ml)	-	-	-	6.25	12.5	25

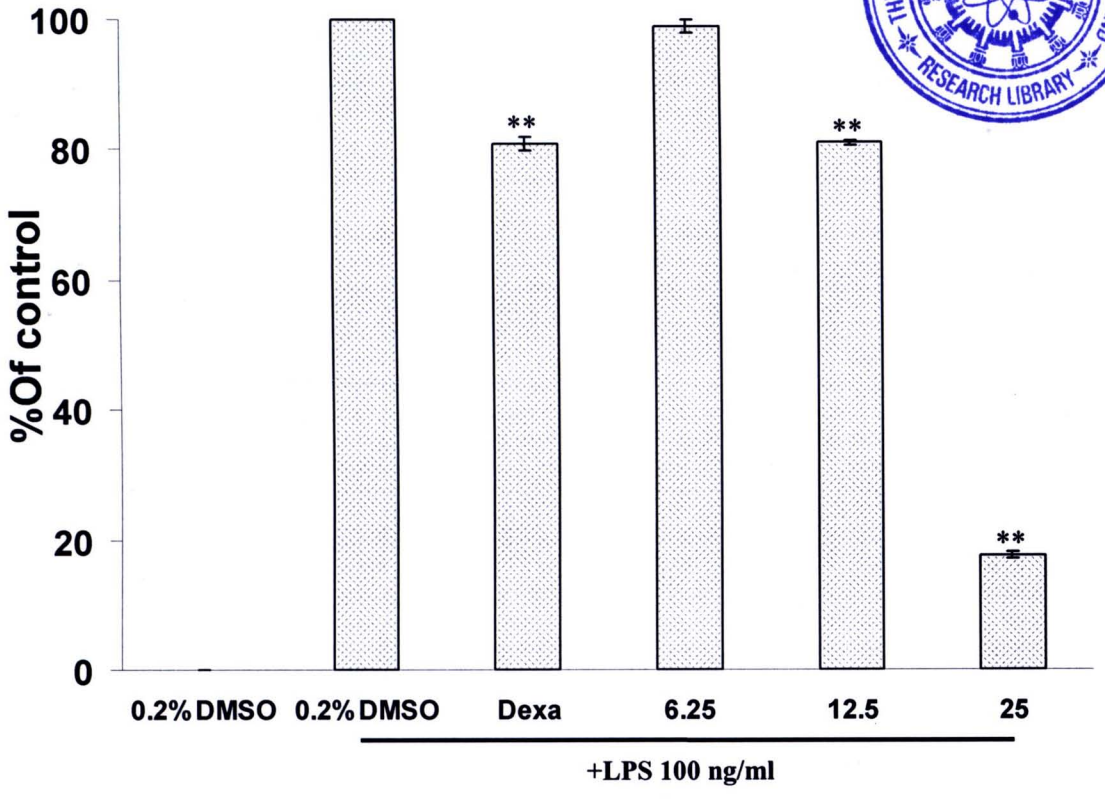
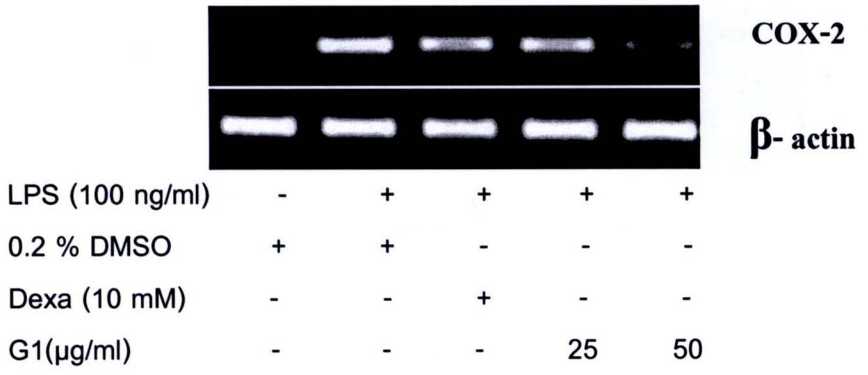


Figure 21 : Effect of G6 on mRNA expressions of iNOS in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).

4. Effect of *Glycosmis parva* extracts on the expressions of COX-2 in LPS stimulated-macrophages.

The inhibitory effects of the extracts on inflammatory mediator, prostaglandins, were also indirectly investigated by determining the expression of COX-2. J774A.1 cells were treated in the same way as the above study for iNOS expression. The total RNA was isolated from the treated cells and used to determine the expression of COX-2 by RT-PCR. All extracts significantly inhibited COX-2 expression except for the low concentration of G5 and G6 (6.25 µg/ml) (Fig.22-25). The prominent inhibition effect on COX-2 expression not only found in hexane and ethyl acetate extracts from the leaves of *G. parva* (G5 and G6) at concentration 25 µg/ml (82.1% and 93% respectively, Appendix B-29 and B-30) but also in hexane extract from branches (G1) at concentration 50 µg/ml (69.92%, Appendix B-27)



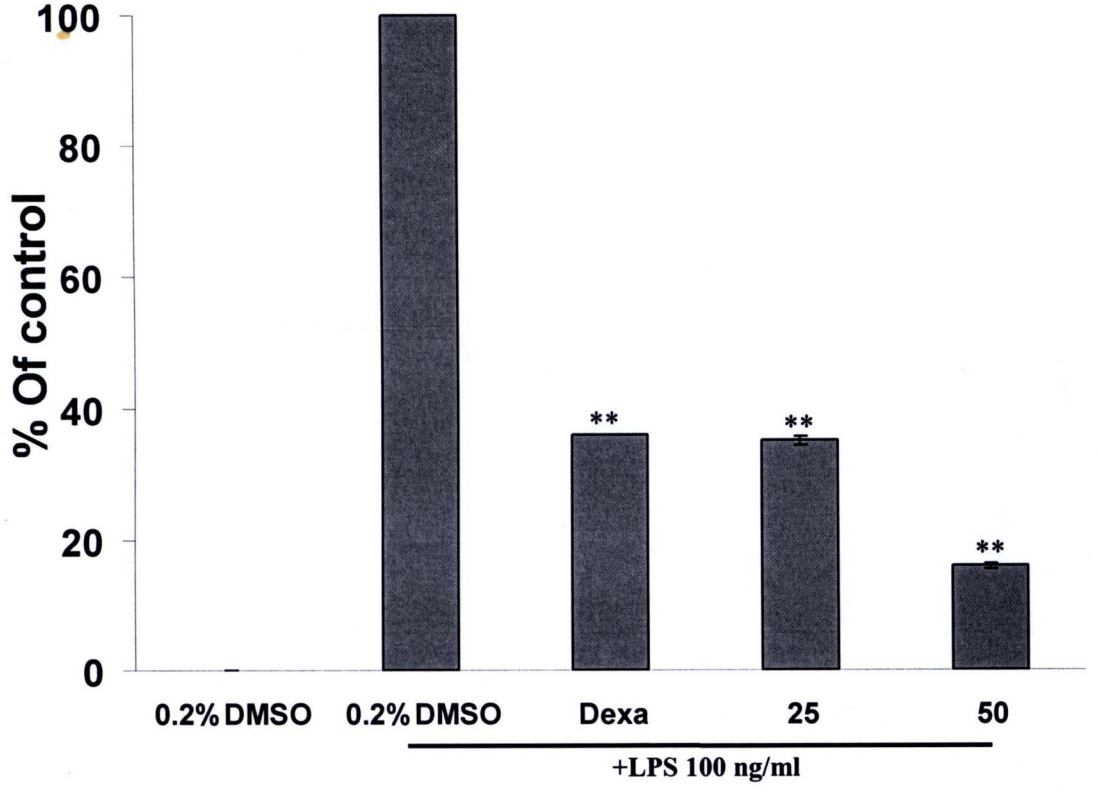
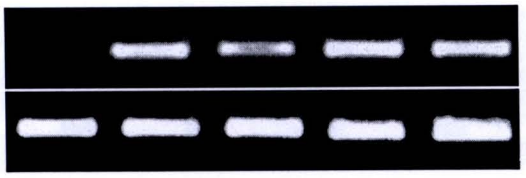


Figure 22 : Effect of G1 on mRNA expressions of COX-2 in LPS stimulated-macrophage J774A.1 cells. ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).



LPS (100 ng/ml)	-	+	+	+	+
0.2 % DMSO	+	+	-	-	-
Dexa (10 mM)	-	-	+	-	-
G2 (μg/ml)	-	-	-	10	20

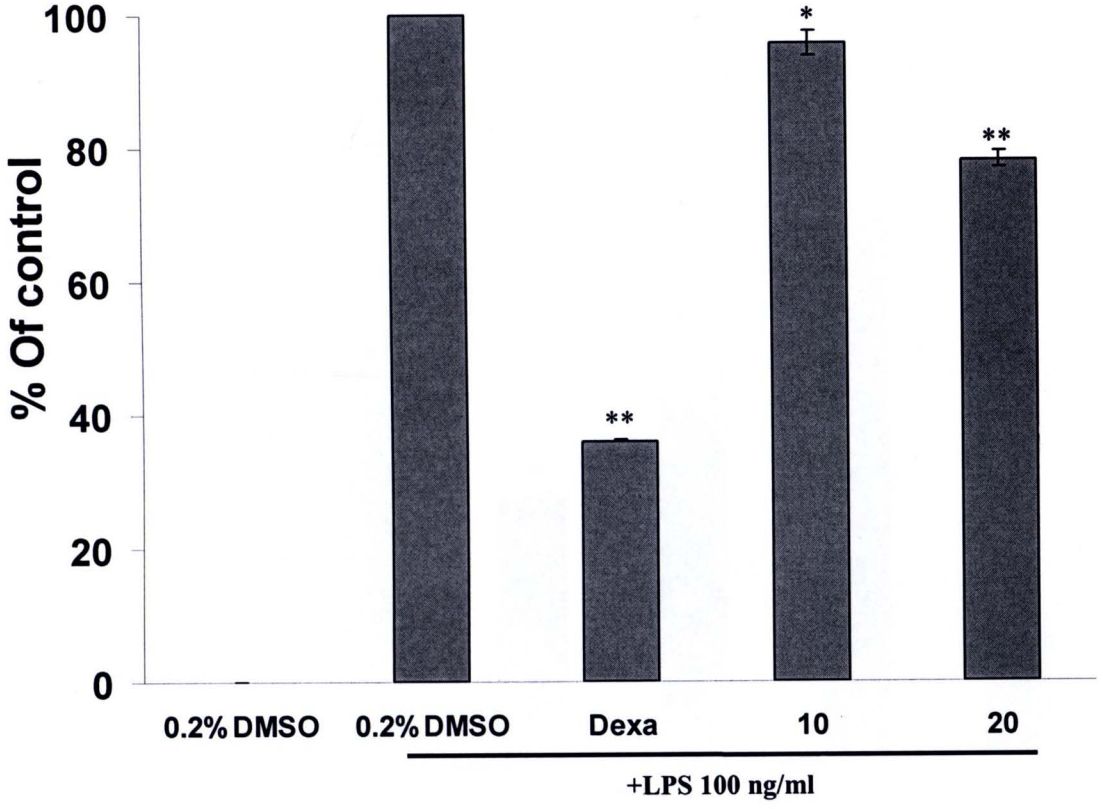
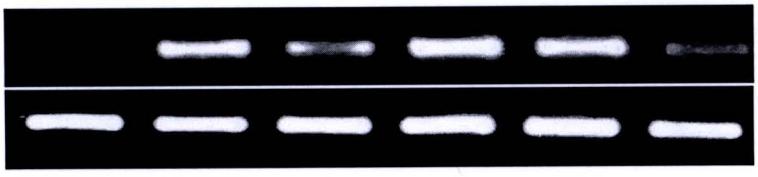


Figure 23 : Effect of G2 on mRNA expressions of COX-2 in LPS stimulated-macrophage J774A.1 cells . * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G5 (μg/ml)	-	-	-	6.25	12.5	25

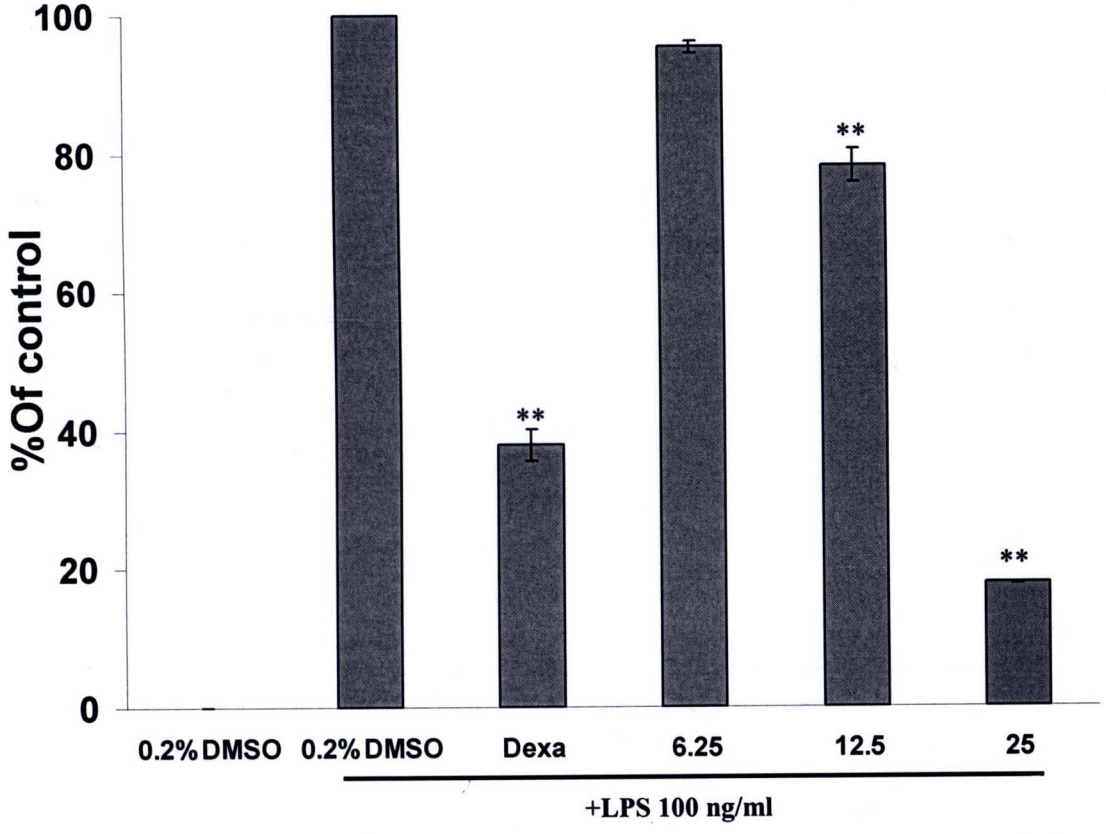
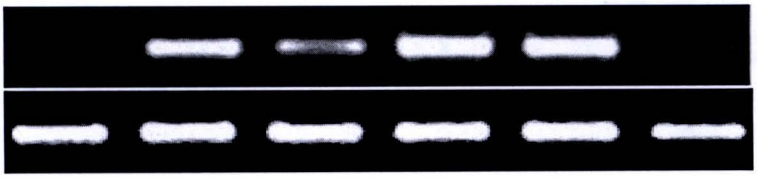


Figure 24 : Effect of G5 on mRNA expressions of COX-2 in LPS stimulated-macrophage J774A.1 cells ; ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$). Results are means ± S.D. (N=2).



LPS (100 ng/ml)	-	+	+	+	+	+
0.2 % DMSO	+	+	-	-	-	-
Dexa (10 mM)	-	-	+	-	-	-
G6 (μg/ml)	-	-	-	6.25	12.5	25

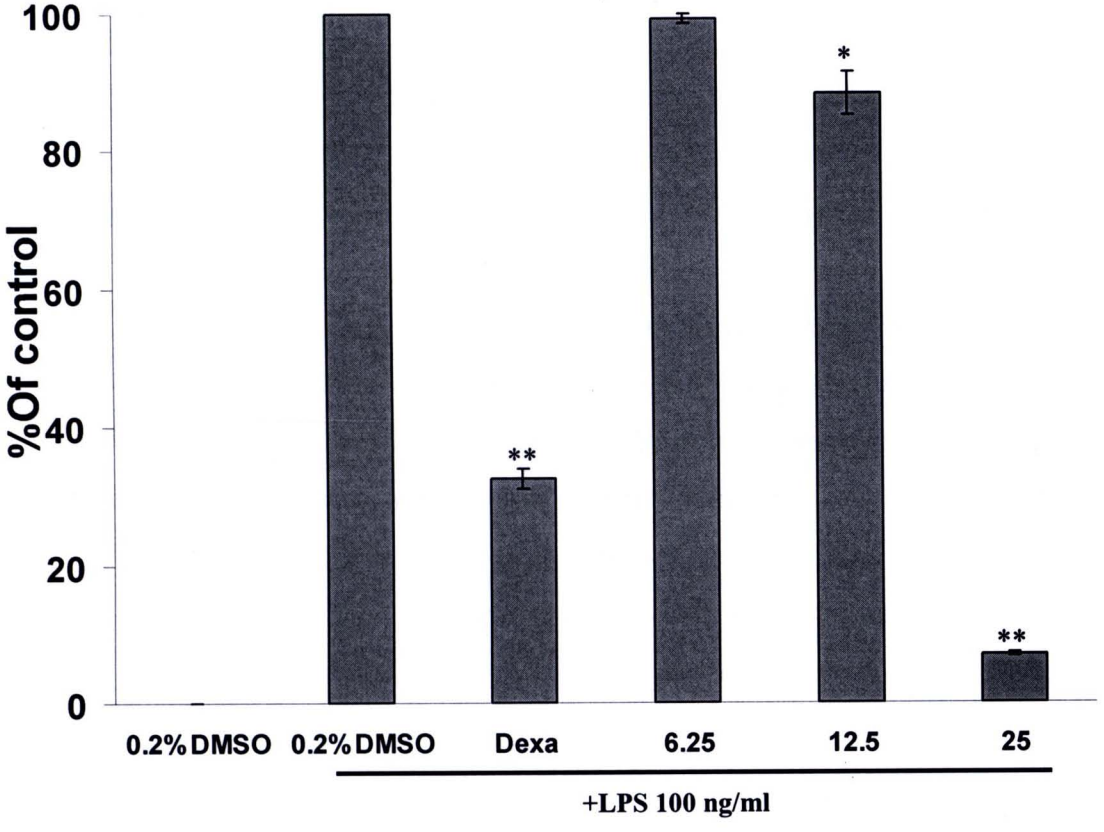


Figure 25 : Effect of G6 on mRNA expressions of COX-2 in LPS stimulated-macrophage J774A.1 cells. * significantly different between 0.2% DMSO+LPS and test compounds ($p<0.01$), ** significantly different between 0.2% DMSO+LPS and test compounds ($p<0.001$) Results are means ± S.D. (N=2).