嘉南藥理科技大學專題研究計畫成果報告

蓮葉抽出物的抗氧化性

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Introduction

Various reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) are generated as by-products during aerobic metabolisms in cells . These highly reactive species have a wide potential for causing cellular components, such as DNA, protein and lipid damages. It is widely acknowledged that the accumulation of oxidative damages of intracellular macromolecules is an essential element in aging processes and in some degenerated diseases .

Herbs have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasonings, beverages, cosmetics, dyes, and medicines. Many active phytochemicals, including the flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins, and phthalides have been identified . Numerous in vitro studies have showed that some of the phytochemicals are potent antioxidants, metal chelators or free radical scavengers and this may go some way to account for their health-promoting properties .

Lotus (Nelumbo nucifera Gertn.) was a popular economic aqueous plant in South East Asia. Every part of lotus, including seeds, rhizomes, flowers, stems and leaves, can be used as food or folk medicine. Lotus leaves traditionally has been used for enhancement of blood circulation, anti-hemorrhages and anti-dizzy, reduction of edema, and recently for weight reduction in Chinese medicine. There has been a relative scarcity of definitive evidence either in vitro or in cultured human cell to prove their biological activity.

In the current study, we investigated the antioxidant activity of the methanol extract of lotus leaves both in vitro and in cell culture systems. Our data suggested that the methanol extract of lotus leaves possesses strong reducing power, chelating ability of metals, as well as scavenging effects of hydroxyl radical and free radicals, thereby inhibiting the oxidation of DNA, protein, and fatty acid in vitro. The methanol extract of lotus leaves in addition showed dose-dependent cytoprotection effect against oxidative damage in HepG2 . No significant cytotoxicity was detected for the extract.

Materials and Methods

Extraction

Lotus leaves (Nelumbo nucifera Gertn.) were harvested from local farm in Tainan. Lotus leaves were extracted with methanol at the ratio of 600 ml per 60 grams and stored at -20°C until used.

Antioxidative activity in a linoleic acid system

The antioxidative activity of methanol extract of lotus leaves was determined by a modified rapid photometric assay (Kuo et al., 1999) and thiobarbituric acid-reactive substances (TBARS) method(Tamura and Shibamoto, 1991).

DNA damage analysis : (1)Plasmid Relaxation Assay(Kobayashi et al., 1990). (2) DNA-methyl green assay for DNA fragmentation (Sinicropi et al., 1994)

Protein oxidativedamage analysisby by DNPH assay (Reznick and Packer, 1994)

Reducing power(Oyaizu, 1986)

DPPH scavenging effect (Shimada et al., 1992)

Scavenging effect of hydroxyl radicals (Halliwell et al., 1987)

Ferrious ion chelating effect (Dinis et al., 1994)

Culture of HepG2 cells

HepG2 cells were purchased from CCRC (Taiwan) and were cultured in Modified Eagle's Medium (MEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 1% non-essential amino acids, 1mM pyruvate, 100U/ml penicillin and 100μg/ml streptomycin (all reagents were obtained from Gibco). Cells were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

Cytotoxicity test of methanol extract of lotus leaves

Different concentrations of methanol extract were incubated with 10^6 HepG2 cells or 10^5 Caco-2 cells in a 6-well plate for 18 hours. MTT assay and LDH leakage assay were employed to determine the cytotoxicity.

MTT assay (Carmichael et al., 1987)

The assay is dependent on the cellular reduction of MTT by the mitrochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically.

LDH leakage assay

LDH activity was determined by commercial kits (Sigma, USA)

Protection against oxidative stress by co-treatment of methanol extract of lotus leaves

Cells were cultured until confluent. Media were aspirated and cells were then washed by PBS once. Various concentrations of extract and 10mM H_2O_2 containing serum free-MEM was added and cultured for 18 hours. The cytotoxicity was then measured as mentioned above.

Protection against oxidative stress by pre-treatment of methanol extract of lotus leaves

Cells were cultured until confluent. Various concentrations of extract were added and culture for various periods. Media were aspirated and cells were washed by PBS once to remove any residual extract. 10mM H_2O_2 containing serum free-MEM was then added and cultured for another 18 hours. The cytotoxicity was then measured as mentioned above.

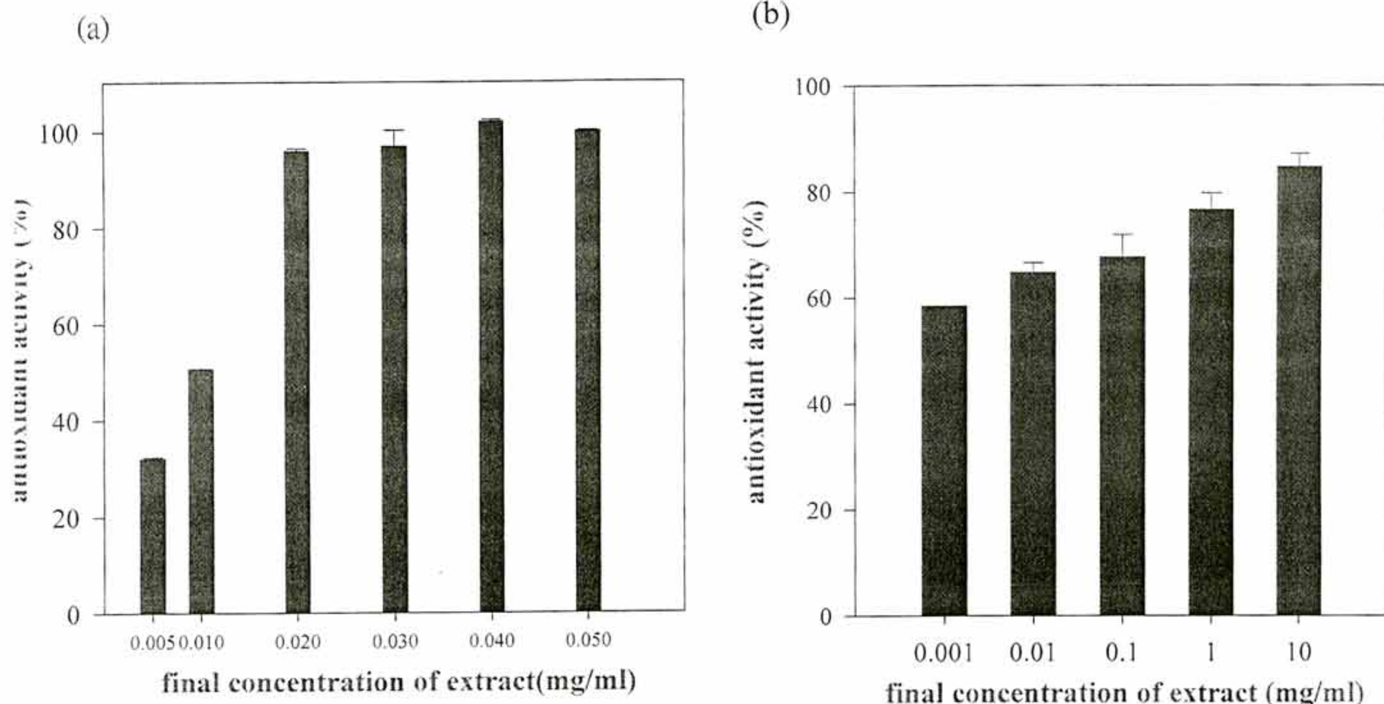


Fig 1. The antioxidant effect of lotus leaves. (a)The antioxidative activity of lotus leaves against linoleic acid peroxidation induced by hemoglobin (b)The antioxidant effect of lotus leaves against linoleic acid peroxidation induced by Fenton reaction

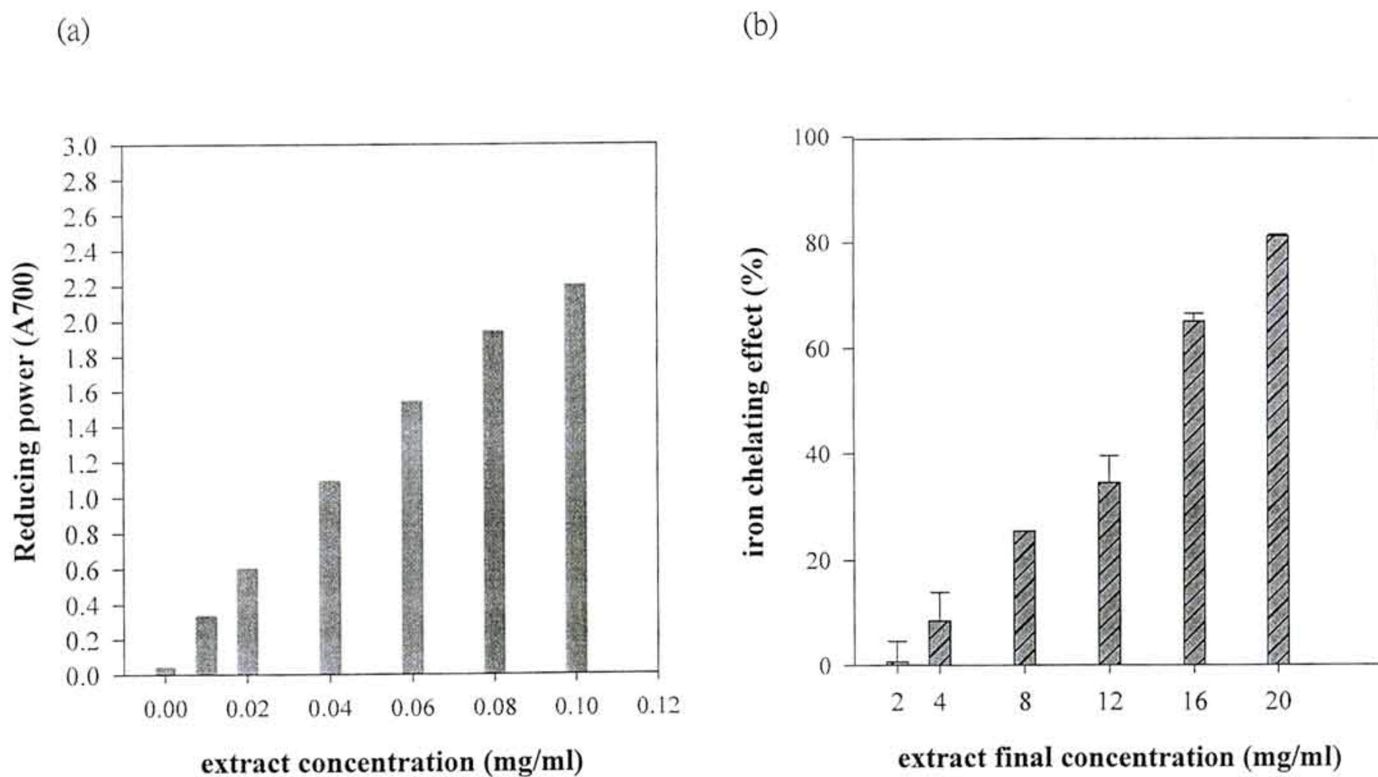
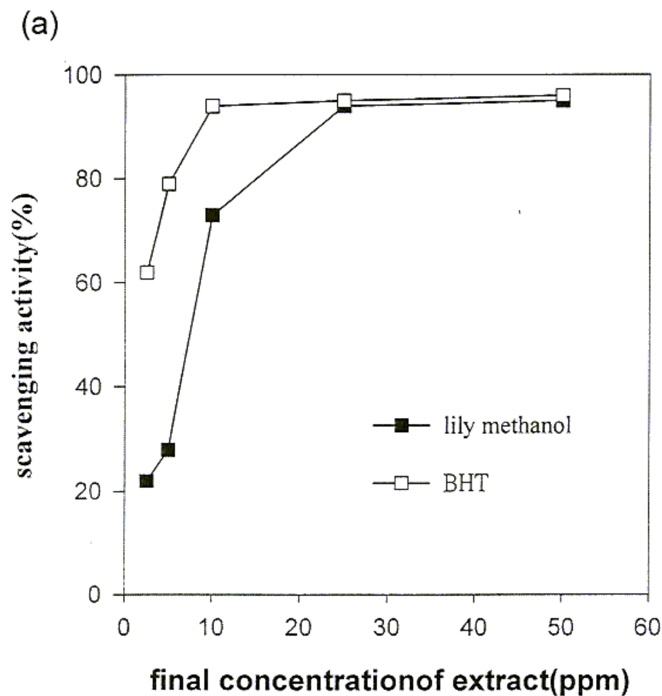


Fig 2. The reducing power and chelating activity on metal ion of lotus leaves extract. (a)The reducing power. (b)The chelating effect of lotus extract on ferrous ion.



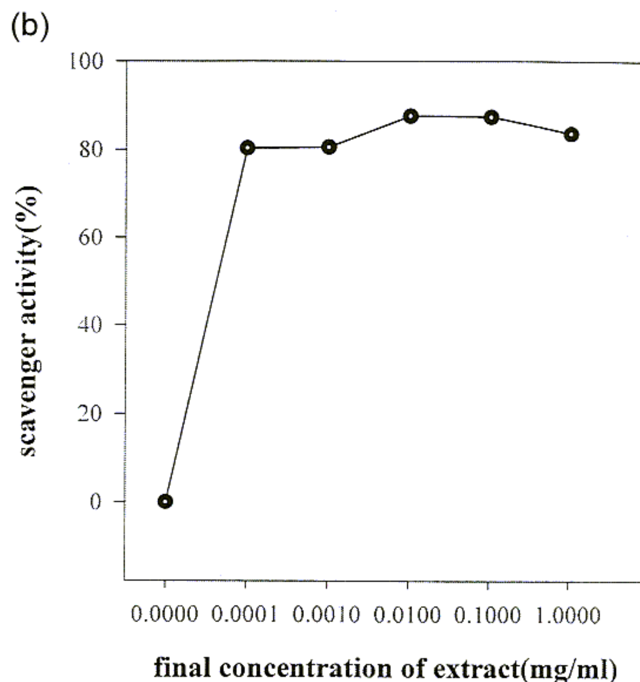
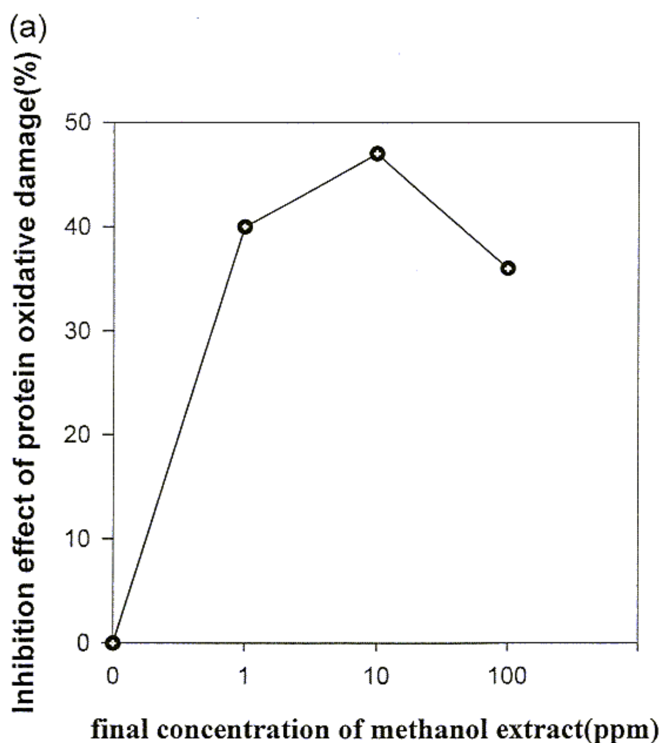


Fig 3. Scavenging effect from various extract of Nulumb nucifera leaves on free radical. (a)Scavenging effect of methanol extract from lotus leaves on DPPH radical. (b)Scavenging effect on hydroxyl radical.



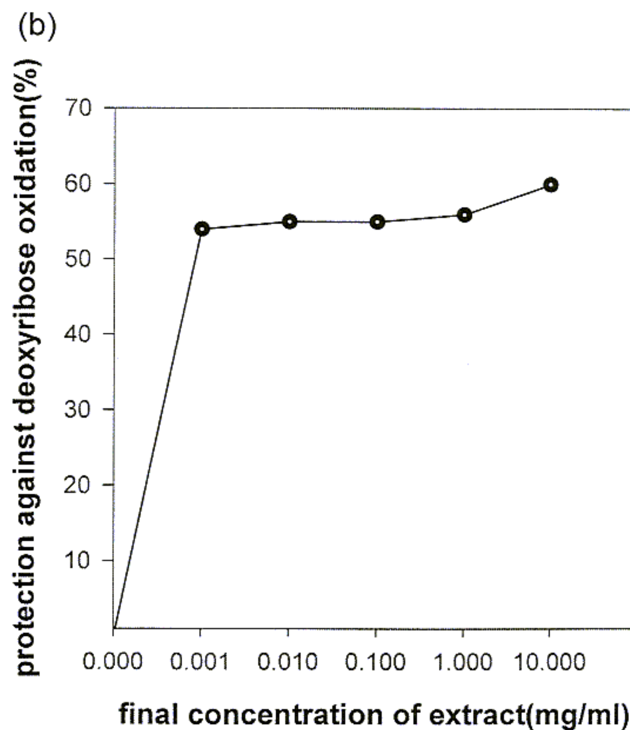


Fig 4. Inhibition effect of protein and deoxyribose damage. (a)Inhibition effect on proteir oxidation. (b)Inhibition effect of methanolic extract of lotus leaves on deoxyribose oxidative damage.

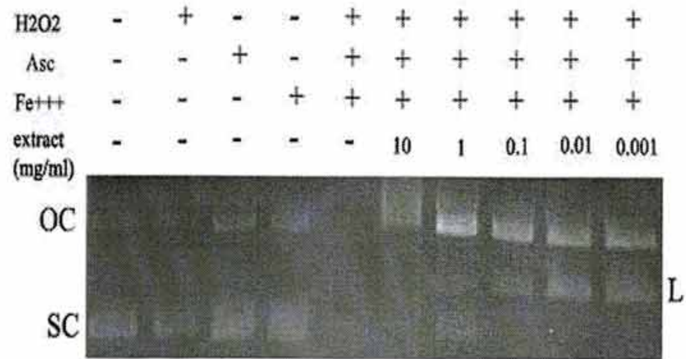


Fig 5. Plasmid relaxing assay. H_2O_2, Fe+++, or ascorbate alone partially damaged DNA and caused a small portion of supercoiled from pUC18 DNA, which migrated fasted, nick to open circular form, which migrated slowest(lane2-4). But in the presence of all three together, DNA was almost completely showen in lane 5. In the presence of extract, the extent of DNA damage could be significantly diminisshed. When extract concentration decreased, the appearence of liner form progressively increased(lanes 6-9).

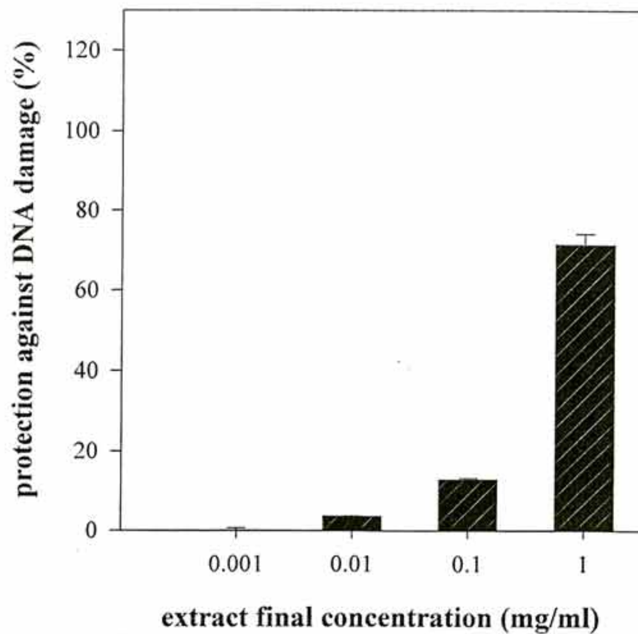


Fig 6. The antioxidant activity of lotus leaves against DNA oxidation. DNA oxidation measured by methyl green method.

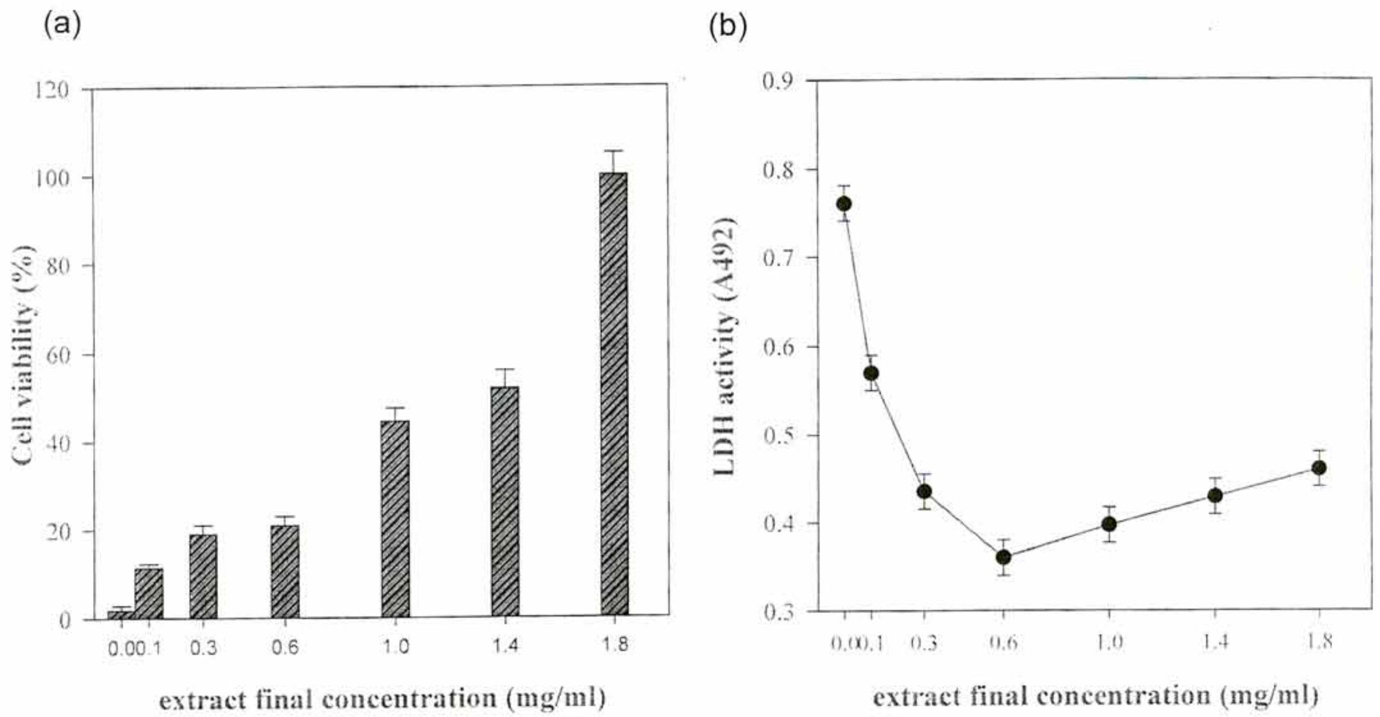


Fig 7. The protective effect of co-treatment of methanol extract of lotus leaves on oxidative damage of HepG2 cell. (a) Detected cell viability by MTT assay. (b) LDH activity.

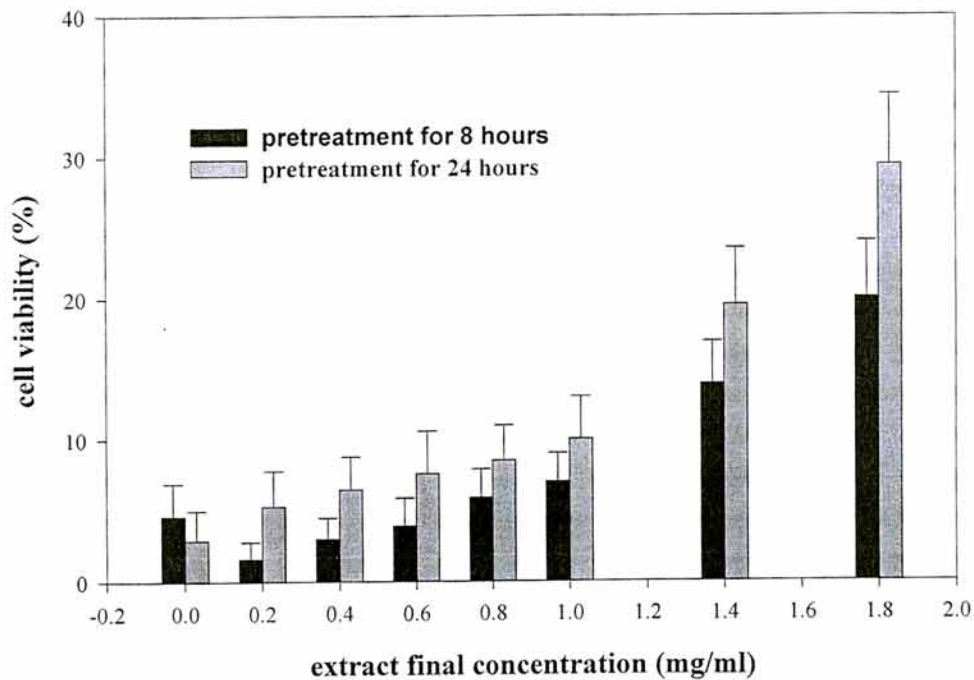


Fig 8. Protection effect of pre-treatment of methanol extract of lotus leaves on oxidative damage of HepG2 cells

Conclusion

Leaves of Lotus (Nelumbo nucifera Gertn.) are commonly used as herbal beverage, food and folk medicine to treat hemorrhages, dizzy, edema and bleeding in Taiwan. In this study, two systems, in vitro and cell culture, were employed to investigate the antioxidant activity of methanol extract of lotus leaves.

The in vitro system showed that the extract exhibited dose-dependent antioxidant activity against linoleic acid peroxidation induced by either Fenton reaction or hemoglobin. The protection effect against oxidative damage of DNA by extract was demonstrated by both plasmid relaxation reaction and methyl green binding assay. Furthermore, the carbonyl content of BSA was significantly lower in the treatment of extract indicating the antioxidant activity of lotus leaves against protein oxidation. The extent of lipid peroxidatin of LDL was also significantly inhibited by addition of extract. Our results also showed that the extract possesses strong reducing power, chelating ability of metals, as well as scavenging effects of hydroxyl radical and free radicals, thereby inhibiting the oxidation of macromolecules in vitro.

The methanol extract of lotus leaves in addition showed dose-dependent cytoprotection effect against oxidative damage in HepG2 cells. No significant mutagenicity or cytotoxicity was detected for the extract. These results suggest that the methanol extract of lotus leaves contains potent antioxidant phytochemicals which may provide significant protection against chronic diseases.

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