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Full Length Research Paper

The protective effects of ginkgo leaf extract on CCl_4-induced liver injury in mice

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Ginkgo leaf extract were studied for their protective effects against liver injury induced using carbon tetrachloride (CCl_4) in mice. Mice were administered CCl_4 by intraperitoneal injection and received a normal diet or normal diet with various Ginkgo leaf extract. Ginkgo leaf extract significantly reduced the liver damage and improving immunity activities by increasing liver index, B lymphocyte transfer index, Natural killer cell activity and phagocytic activity and decreasing liver IL-1β and TNF-α levels in a dose dependent manner. These results suggested that Ginkgo leaf extract may protect the liver against CCl_4-induced injury by enhancing immunity activity.

Key words: Ginkgo leaf extract, mice, IL-1β, natural killer cell activity, B lymphocyte transfer index, immunity.

INTRODUCTION

With *Ginkgo biloba* achieving unprecedented popularity over the past decade, and the recognition of the important therapeutic effects shown by this plant, there is a growing market for phytomedicines based on its extracts. Many data support the efficacy of *G. biloba* extracts in biological systems, including *in vitro* and *in vivo* experiments and its therapeutic efficacy was also observed in clinical trials of elderly patients and patients with neurodegenerative diseases (Droy-Lefaix, 1997; Akiba et al., 1998; Lugasi et al., 1999; Wei et al., 2000; Kim, 2001; Bush, 2002; Maynard et al., 2002). In ageing processes, *G. biloba* may ameliorate the mitochondria respiratory chain function by quenching the superoxide anion, and the hydroxyl and peroxyl radicals. It protects the brain by facilitating the uptake of neurotransmitters and by reducing ischemia–reperfusion episodes and level of apoptosis (Droy-Lefaix, 1997). Some clinical data exist, that *G. biloba* extracts might be used as an effective drug for the treatment of neuronal diseases associated with the production of peroxynitrite (Wei et al., 2000). Carbon tetrachloride (CCl_4) is an extensively used industrial solvent, and it is the best-characterized animal model of xenobiotic-induced free radical-mediated hepatotoxicity (Recknagel and Glende, 1973).

CCl_4 is an injury agent for animal experiment, which induced reactive oxygen formation and depleted GSH of phase II enzyme. Liver regulates many important metabolic functions. Hepatic injury is associated with distortion of these metabolic functions (Wolf, 1999). In absence of a reliable liver protective drug in the modern system of medicine, a number of medicinal preparations in Ayurveda, the Indian system of medicine, are recommended for the treatment of liver disorders (Chatterjee, 2000).

Natural remedies from medicinal plants are considered to be effective and safe alternative treatments for hepatotoxicity.

In view of this, the present study was undertaken to investigate the hepatoprotective activity of Ginkgo leaf extract against CCl_4 induced hepatotoxicity in mice.

MATERIALS AND METHODS

Ginkgo leaf extract

Ginkgo leaf (400 g) was extracted in boiling water. Then combined extract was condensed and dried under vacuum.

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Table 1. Effect of Ginkgo leaf extract on liver index (mg/g).

Group	Liver index
Control	62.08±2.54
Model	47.29±1.09b
GLE (100 mg/kg B.W.)	53.03±1.79c
GLE (300 mg/kg B.W.)	57.15±2.43d
GLE (500 mg/kg B.W.)	61.03±2.17d

[b]$P<0.01$, compared with control group; [c]$P<0.05$, and [d]$P<0.01$, compared with model group

Experimental procedure

Animals were divided into five groups, each group containing six animals. Group I (normal control) received distilled water for 5 days. Group II (induction control) received CCl_4 3 ml/kg, s.c., 1:1 dilution with olive oil (Lin et al., 1998) on 3rd day. Groups III to V received Lithospermum erythrorhizon extract (100, 300 and 500 mg/kg B.W.) respectively for 5 days and CCl_4 induction on 3rd day. On the 6th day, the animals were sacrificed under ether anesthesia, and blood and liver samples were collected. The blood was allowed to clot for 30 min; serum was separated by centrifuging at 37°C and was used for biochemical estimations. For histopathological study, liver tissue was quickly removed after autopsy and fixed in 10% formosaline.

TNF-α and IL-1β levels and B lymphocyte transfer rate

TNF-α and IL-1β levels were detected by rat TNF-α and IL-1β ELISA kits according to the procedures provided by the manufacturer. The sensitivity of the assay was 5 pg/ml for TNF-α and 5 pg/ml for IL-1β. B lymphocyte transfer rate (%) was measured according to the literature.

Natural killer cell activity

The activity of NK cells was assayed by the chromium release assay as described (Bleavins et al., 1995; Kouhpayeh et al; 2010; Meng et al; 2011). Single cell suspensions were adjusted to four concentrations: 10^7, 5×10^6, 2.5×10^6, and 1.25×10^6 cells/ml. The target cells (106/ml), 51Cr-YAC-1 cells (ATCC, Rockville, MD), were added to each well of a 96-well plate in a volume of 0.1 ml. The effector cells in a volume of 0.1 ml were added to each of the two replicate wells of target cells to obtain effector: target ratios of 100:1, 50:1, 25:1 and 12.5:1. The spontaneous release and the maximum release were determined by adding 0.1 ml of medium and Triton X-100 (0.1%) to each of the 12 replicate wells containing the target cells, respectively. Following a 4 h incubation, the plates were centrifuged, and 0.1 ml of the supernatant was removed from each well and counted using a gamma counter. The mean percent cytotoxicity at each effector concentration was determined.
The lytic units (LU) per 10^7 cells and per spleen were also calculated with one LU defined as the number of effector cells required to lyse 10% of target cells in 4 h.

Assay of phagocytic activity

The function of phagocytosis was investigated by detecting the number of cells that ingested at least one fluorescent particle, according to the method of Kotani et al. (1998). 1×10^6 macrophages were grown in 12-well tissue culture plates overnight. After treatment with LPS, TLR4 siRNA, or a combination of TLR4 siRNA and LPS in an atmosphere of 5% CO_2, macrophages were trypsinized and suspended in PBS. Then, macrophages were incubated at 37°C on a shaking platform. Red fluorescent FluoSphere carboxylate-modified microspheres (molecular probes, Eugene, OR), 0.5 μm in diameter, were added to the cell suspension and incubated for 20 min. The ratio of particle-to-cell was 15:1.

The reaction was inhibited by an ice-cold saline solution. The numbers of macrophages that ingested at least one fluorescent particle were counted with the aid of a crosshair micrometer (Nikon).

Statistical analysis

All the data were statistically analyzed by one-way ANOVA (SPSS, 1999). Differences among treatments were separated by Duncan's multiple range tests. Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

The liver is the largest gland in the body and performs an astonishingly large number of tasks that impact all body systems. It has a wide range of functions, including detoxification, protein synthesis, and production of biochemicals necessary for digestion (Angelico et al., 2005; Maliwichi-Nyirenda and Maliwichi, 2010). The liver is necessary for survival; there is currently no way to compensate for the absence of liver function long term, although liver dialysis can be used as short term (Roche and Samuel, 2008; Petta et al., 2009; Chen et al., 2011). This organ plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. It lies below the diaphragm in the abdominal-pelvic region of the abdomen. It produces bile, an alkaline compound which aids in digestion via the emulsification of lipids. The liver's highly specialized tissues regulate a wide variety of high-volume biochemical reactions, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions (Anthea et al., 1993).

In the present study, liver index was markedly reduced in CCl_4-induced mice. Pretreatment with Ginkgo leaf extract (100, 300, or 500 mg/kg body weight) significantly suppressed the decrease of liver index in a dose-dependent manner (Table 1).

These results indicate that Ginkgo leaf extract is a potent hepatoprotective agent against CCl_4-induced injury. IL-1β is a member of the interleukin 1 cytokine family. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation,

Table 2. Effect of Ginkgo leaf extract on liver IL-1β and TNF-α levels in CCl_4-induced mice.

Group	IL-1β	TNF-α
Control	158.32±3.29	69.43±2.15
Model	237.54±4.11b	97.68±3.09b
GLE (100 mg/kg B.W.)	209.57±5.18d	88.56±2.68c
GLE (300 mg/kg B.W.)	186.48±3.94d	76.45±2.22d
GLE (500 mg/kg B.W.)	163.22±4.03d	70.41±2.83d

[b]$P<0.01$, compared with control group; [c]$P<0.05$, and [d]$P<0.01$, compared with model group.

Table 3. Effect of Ginkgo leaf extract on B lymphocyte transfer index, natural killer cell activity and phagocytic activity in CCl_4-induced mice.

Group	B lymphocyte transfer index	Natural killer cell activity (%)	Phagocytic activity
Control	1.63±0.09	95.34±2.74	5.23±0.24
Model	0.85±0.02b	64.39±2.61b	3.04±0.11b
GLE (100 mg/kg B.W.)	1.06±0.08d	78.41±3.09d	3.92±0.13d
GLE (300 mg/kg B.W.)	1.31±0.05d	83.77±4.17d	4.78±0.17d
GLE (500 mg/kg B.W.)	1.59±0.08d	90.38±3.53d	5.14±0.27d

[b]$P<0.01$, compared with control group; and [d]$P<0.01$, compared with model group.

differentiation and apoptosis (Boraschi et al., 2009; Okamura et al., 1999). Tumour necrosis factor-alpha (TNF-α) is a multi-functional cytokine that can regulate many cellular and biological processes such as immune function, cell differentiation, proliferation, apoptosis and energy metabolism. It is synthesised as a 26-kDa transmembrane monomer (mTNF-α) (Kriegler et al., 1988) that undergoes proteolytic cleavage by the TNF-a converting enzyme (TACE) to yield a 17-kDa soluble TNF-a molecule (sTNFα) (Black et al., 1997). Both sTNF-a and mTNF-a can effect biological and metabolic responses (Perez et al., 1990; Xu et al., 1999) suggesting that mTNF-α may mediate paracrine and autocrine signals, leaving sTNF-α to mediate endocrine effects (Grell, 1995). However, more recent indications are that the endocrine actions of sTNF-α depend on the maintenance of high circulating levels, which are more likely to occur in catabolic disease states such as the cachectic conditions associated with sepsis and cancer.

The liver IL-1β and TNF-α levels in CCl_4-induced mice was used as a biochemical marker for hepatic injury. Liver IL-1β and TNF-α levels in model mice were significantly higher than those in normal control. Treatment with Ginkgo leaf extract (100, 300, or 500 mg/kg body weight) produced a significant increase in liver IL-1β and TNF-α levels of experimental mice (Table 2). These results suggest that Ginkgo leaf extract can significantly ($P<0.01$) attenuate liver IL-1β and TNF-α levels in a dose-dependent manner. B lymphocytes are also called B cells. A type of lymphocyte that circulates in the blood and lymph and produces antibodies when it encounters specific antigens. Natural killer (NK) cells are considered important for the host defence against neoplasia and the establishment of infections (Trinchieri, 1989). These diseases occur with increasing incidences in elderly subjects (Crossley and Peterson, 1996). Low NK cell activity has been associated with death due to infections in elderly humans (Ogata et al., 1997; Lv et al., 2010). Furthermore, middle-aged humans had decreased NK cell activity compared to young controls, whereas NK cell activity of centenarians was in the range of the young group (Sansoni et al., 1993). This finding formed the basis for the hypothesis that well-preserved NK cell activity is important for successful aging (Franceschi et al., 1995).

Phagocytosis in mammalian immune cells is activated by attachment to pathogen-associated molecular patterns (PAMPS), which leads to NF-κB activation. Opsonins such as C3b and antibodies can act as attachment sites and aid phagocytosis of pathogens (the immune system). Phagocytosis process the human body uses to destroy dead or foreign cells. Macrophages are the scavenger cells that are part of this process. This works reasonably well for whole bacteria or viruses, but less so for proteins or encapsulated bacteria. In order to deal more effectively with encapsulateed bacteria, antibodies directed against the capsule enable the phagocytic cells to ingest the organisms, using their Fc receptors. The results of liver B lymphocyte transfer index, natural killer cell activity and phagocytic activity in CCl_4-induced mice are shown in Table 3. The model mice had a significantly ($P<0.01$) lower B lymphocyte transfer index, natural killer cell activity and phagocytic activity than the control group.

On day 7, the administration of Ginkgo leaf extract

enhanced significantly dose-dependently B lymphocyte transfer index, natural killer cell activity and phagocytic activity ($P<0.01$) in liver of CCl_4-induced mice.

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