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Mangosteen peel extract reduces formalin-induced liver cell death in rats

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ABSTRACT

BACKGROUND

Formalin is a xenobiotic that is now commonly used as a preservative in the food industry. The liver is an organ that has the highest metabolic capacity as compared to other organs. Mangosteen or *Garcinia mangostana* Linn (GML) peel contains xanthones, which are a source of natural antioxidants. The purpose of this study was to evaluate the effect of mangosteen peel extract on formalin-induced liver cell mortality rate and p53 protein expression in Wistar rats.

METHODS

Eighteen rats received formalin orally for 2 weeks, and were subsequently divided into 3 groups, consisting of the formalin-control group receiving a placebo and treatment groups 1 and 2, which were treated with mangosteen peel extract at doses of 200 and 400 mg/kgBW/day, respectively. The treatment was carried out for 1 week, and finally the rats were terminated. The differences in liver cell mortality rate and p53 protein expression were analyzed.

RESULTS

One-way ANOVA analysis showed significant differences in liver cell mortality rate among the three groups (p=0.004). The liver cell mortality rate in the treatment group receiving 400 mg/kgBW/day extract was lower than that in the formalin-control group. There was no p53 expression in all groups.

CONCLUSIONS

Garcinia mangostana Linn peel extract reduced the mortality rate of liver cells in rats receiving oral formalin. Involvement of p53 expression in liver cell mortality in rats exposed to oral formalin is presumably negligible.

Key words : Formalin, mangosteen peel, cell death, p53, rats

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Ekstrak kulit manggis menurunkan kematian sel hepar tikus wistar yang diinduksi formalin

ABSTRAK

LATAR BELAKANG

Formalin merupakan salah satu xenobiotik yang sekarang ini banyak ditemukan dalam industri makanan sebagai pengawet. Hepar merupakan organ yang memiliki kapasitas metabolik tertinggi dibanding organ lain. Kulit manggis atau Garcinia Mangostana Linn (GML) mengandung xanton yang merupakan sumber antioksidan. Tujuan penelitian ini adalah untuk menilai pengaruh ekstrak kulit GML terhadap jumlah kematian dan ekspresi protein p53 sel hepar tikus wistar yang diinduksi larutan formalin.

METODE

Delapan belas ekor tikus diberikan formalin peroral selama 2 minggu. Tikus secara acak dibagi menjadi 3 kelompok. Kelompok pertama sebagai kelompok kontrol (K) diberi placebo, kelompok kedua (Perlakuan 1) dan kelompok ketiga (Perlakuan 2) masing-masing diberi ekstrak kulit manggis 200 dan 400 mg/kgBB/hari. Perlakuan diberikan selama 1 minggu dan diakhiri dengan terminasi tikus. Penilaian sel hepar dilakukan dengan melihat dan menghitung sel mati berdasarkan perubahan struktur histopatologi sel hepar, yaitu yang ditandai dengan adanya salah satu dari inti yang piknotik, karioreksis maupun kariolisis. Pemeriksaan ekspresi p53 dilakukan dengan melihat dan menghitung sel hepar yang positif terekspresi p53, yaitu sel dengan inti dan sitoplasma yang tercat coklat.

HASIL

Analisis one way anova *menunjukkan perbedaan jumlah kematian sel hepar pada ketiga kelompok (p=0,004). Jumlah kematian sel hepar kelompok perlakuan dosis 400 mg/kgBB/hari ternyata lebih rendah dibanding kelompok kontrol. Ekspresi p53 tidak didapatkan pada ketiga kelompok.*

KESIMPULAN

Ekstrak kulit manggis mampu menurunkan jumlah kematian sel hepar tikus yang dipaparkan formalin secara oral. Kemungkinan adanya keterlibatan p53 pada kematian sel hepar tikus yang dipaparkan formalin secara oral sangat kecil.

Kata kunci : *Formalin, kulit manggis, kematian sel, p53, tikus*

INTRODUCTION

Formalin is a xenobiotic that is now commonly found in the food industry as a preservative. The concentrations used are frequently unknown, because they are not registered with and not monitored by the Department of Health and the local Drug and Food Supervisory Board (BPOM). A survey carried out by BPOM demonstrated the existence of a number of formalin-containing foods, such as raw noodles, meat balls, dried salted fish, and tofu.[1]

Formalin is a colorless liquid with a pungent odor, being a solution of approximately 37 percent formaldehyde in water, to which up to 15 percent methanol is added as a preservative. Exposure to formaldehyde takes place by 4 routes, i.e. by inhalation and ingestion, and through the skin and eyes. Formaldehyde

entering the body by inhalation or ingestion is rapidly metabolized, but rather slowly when entering through cutaneous or ocular exposure.[1]

Formaldehyde entering the body in amounts above toxic threshold levels will react with nearly all substances in the cells, depressing cellular functions, and ultimately causing organ damage.[2] Unmetabolized formaldehyde may react with tetrahydrofolate, producing single carbon atoms that are electrophilic and react strongly with macromolecules, including DNA and proteins. Single carbon atoms also react strongly with nucleophilic cell membranes, causing increased production of reactive oxygen species (ROS) in the body, thus resulting in oxidative stress. Oxidative stress may cause peroxidation reactions of membrane lipids, oxidation of proteins, including enzymes, and oxidation of DNA, leading to oxidative damage and carcinogenesis.[3-5]

Several studies have demonstrated the influence of formalin in cellular damage. Formalin administered to rats at a dose of 100 ppm for 1 week will cause damage to cell membranes, marked by increased malondialdehyde (MDA) and morphologic changes in hepatic tissues, as determined by histopathology. The free radicals produced by formaldehyde, particularly hydroxyl radicals (OH-), cause peroxidation of unsaturated fatty acids in cell membranes, resulting in oxidative damage of liver cells.[2] Repeated exposure of mice to fish containing formalin at oral doses of 0.2 and 0.5 ppm for 3 months resulted in focal necrosis of the liver and renal tubuli.[6]

Hepatic cells undergoing cellular stress may be detected by immunohistochemical determination of p53 protein expression. The p53 proteins are produced in the cells as monitors of biochemical signals that may indicate DNA damage and mutations.[7] As a result, p53 may induce cell cycle arrest at G0 (preventing the cells from continuing the cell cycle, to give time for the DNA to perform repairs), or cause apoptotic cell death.[7-9] Expression of p53 is frequently used in various studies as indicator of cellular damage and cell death.

Mangosteen or *Garcinia mangostana* Linn is a tropical plant native to Indonesia (where it originated from Kalimantan) and to other Southeast Asian countries. Mangosteen peel extracts have been extensively utilized by communities, especially in Thailand, for treatment of wound infections, amebic dysentery, and as anti-inflammatory agents.[10] Mangosteens are fruits that have been called super fruits, because they have a nice taste, are rich in nutrients, have antioxidant effects, and may reduce the risk of illness in humans.[11]

Mangosteen peel has been demonstrated to contain polyphenols, which are phenolic compounds with natural antioxidant properties. Phenolic compounds are organic compounds possessing at least one aromatic ring with one or more hydroxyl groups. They may exert antioxidant effects because of their ability to stabilize free radicals, by rapidly delivering a hydrogen atom to the free radicals. In this way, phenolic compounds may protect cells from oxidative damage.[12,13]

One study stated that the compound alpha-mangostin from mangosteen peel extract inhibited cell death from cisplatin, and reduced ROS production and p53 expression.[14] Other studies mentioned that alpha-mangostin decreased tumor growth and metastases in mammary carcinoma.[15] Many studies have explained the antioxidant effects of mangosteen peel on the basis of its hydroxyl and superoxide radical scavenging property.[10] However, there have been no studies on the effects of mangosteen peel on cell death resulting from formalin-induced chemical lesions. The objective of this study was to evaluate the effect of mangosteen peel extract on p53 protein expression in liver cells and on the mortality rate of Wistar rat liver cells after induction by formalin.

METHODS

Design of the study

This was an experimental laboratory study of post test control group design using Wistar rats as experimental subjects. The study was carried out in September 2013 for a period of 3 weeks. The study setting was at the Inter University Center *(Pusat Antar Universitas)* of Gadjah Mada University (PAU UGM), Yogyakarta, and the Pathologic Anatomy laboratory of Diponegoro University (UNDIP), Semarang.

Experimental animals

The male Wistar rats used in this study were obtained from Gajah Mada University, Yogyakarta, according to the following inclusion criteria: weight 150-200 grams, age 12 weeks, in healthy and active condition, and with normal external anatomic features. Exclusion criteria: rats dying or becoming ill during the study. The minimum number of animals required for each group was 5, thus for 3 groups 15 animals were needed. In anticipation of dropouts one animal was added to each group. The sample size were according to the Research Guidelines for Evaluation the Safety Efficiency of Herbal Medicines in World Health Organization (WHO).[16] So in this study there were 6 animals in each group.

Preparation of mangosteen peel extract

Mangosteen peel (pericarp) was cleansed, cut up into small parts, covered with black cloth and left to dry thoroughly in the sun. The dried peel was then minced in a blender, and 100 grams dry weight of the peel was placed in a 1-liter Erlenmeyer flask. After addition of 96% ethanol to a volume of 900 ml, the mixture was thoroughly stirred, then left overnight until all particulate matter had settled to the bottom of the flask. The supernatant containing the solvent was then passed through filter paper. The filtrate was placed in a 1-liter evaporation flask connected to an evaporator, and the water bath was completely filled with water. The complete setup, including the rotary evaporator and water bath (set at 90°C), was then connected to the mains electricity. The ethanolic solution was left in the evaporator to separate from the active substances already present in the flask, until the ethanol had stopped dripping into the receiving flask (1.5 to 2 hours for 1 flask). The extract was then put in plastic bottles and freeze-dried.

Intervention

The rats were adapted to feed for 1 week. The animals were kept in a room at a temperature of 25 ± 2 °C and 65-70% humidity, with a 12-hour light and dark cycle. Formalin was then administered for 21 days, after which the rats were randomly assigned to 3 groups. The grouping was done by simple random allocation to prevent bias due to variation in age and weight. The formalin-control group received a placebo, while treatment groups 1 and 2 received mangosteen peel extract by gavage at a dose of 200 mg/kgBW/day and 400 mg/kgBW/day, respectively, both placebo and extract being given for 1 week. The rats were terminated by cervical dislocation under anesthesia after 1 week of treatment.

Histological analysis

The liver of each rat was measured and weighed, macroscopically examined and fixed in a vessel containing 10% buffered formalin, at a ratio of 1 part liver tissue and 9 parts 10% buffered formalin. The liver tissue was then processed by standard histological technique and stained with hematoxylin-eosin. The liver cells were examined histologically for structural changes, followed by counting those cells showing either pycnotic nuclei, karyorhexis, or karyolysis. The evaluation and cell count results were compared with those of our pathologist, and in case of differing results, a consensus was reached.

Immunohistochemistry of p53

Immunohistochemical staining was performed by the indirect method, to visualize cells with p53 protein expression. Unstained liver slide preparations were ligated with p53 monoclonal antibody and peroxidase-labeled secondary antibody, then characterized by double staining with diaminobenzidine (DAB) and hematoxylin-eosin. The slides were then examined and counted for cells with positive p53 expression, showing brown nuclei and cytoplasms. The evaluation and cell count results were compared with those of our pathologist, and in case of differing results, a consensus was reached.

Statistical analysis

The collected data were processed with the SPSS computer program. The data were evaluated for normality by means of the Saphiro Wilk test. Since the data in this study were normally distributed, testing for between-group differences was done by one-way ANOVA, followed by post-hoc Tukey HSD. The level of significance was set at 0.05.

Ethical clearance

This study was carried out after obtaining ethical clearance from the Commision for Medical Research Ethics, Faculty of Medicine, Diponegoro University, Semarang.

RESULTS

The consensus results of the immunohistochemical assessment were negative for p53 expression, since no brown-colored cells were found, both in the formalin-control group and the treatment groups (Figures 1A, B, and C).

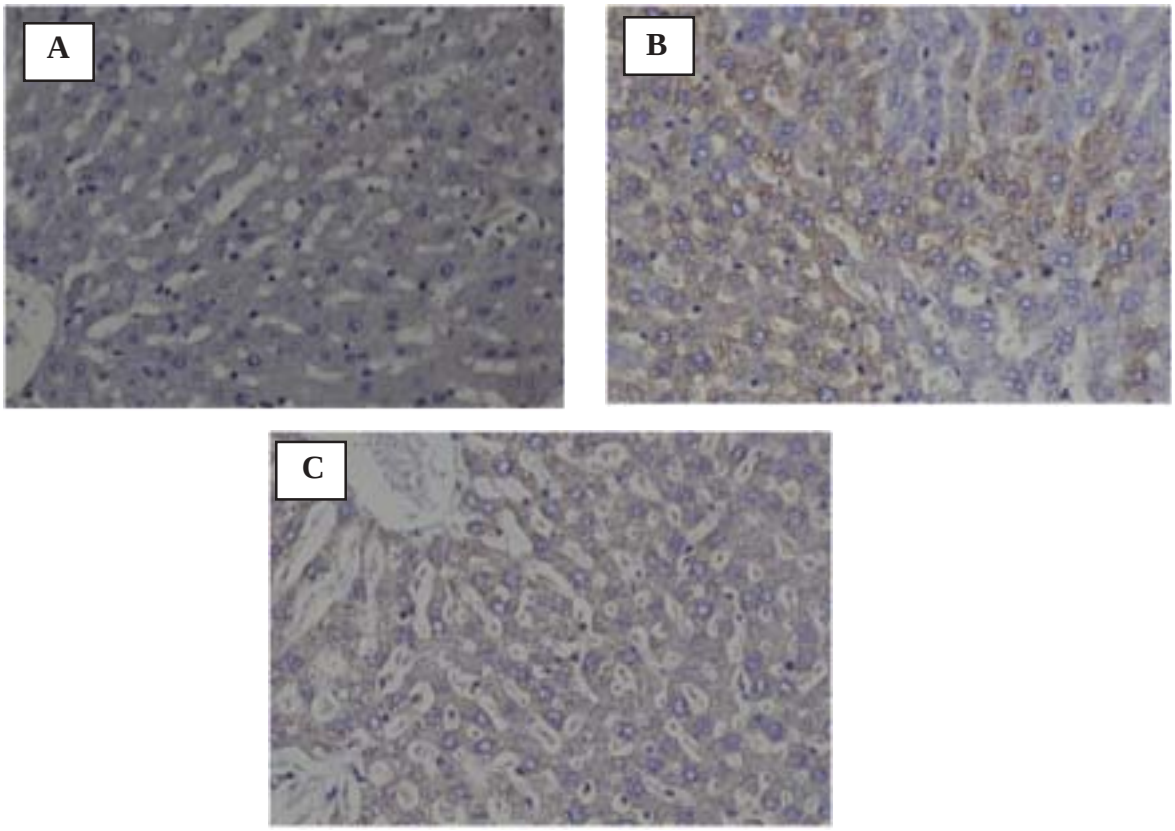

Figure 1. Microscopic views of p53 expression in the three groups, viz. formalin-control group (A), treatment group 1 (B) and treatment group 2 (C). No brown-colored cells were found in the three groups (magnification 400x)

Table 1. Differences in liver cell mortality rate between groups

	Control	Treatment 1	Treatment 2
Control	-	p=0.398	p=0.003*
Treatment 1	p=0.398	-	p=0.046*
Treatment 2	p=0.003*	p=0.046*	-

Treatment 1 : mangosteen peel extract at 200 mg/ kgBW/ day; Treatment 2 : mangosteen peel extract at 400 mg/kgBW/day; *significant p value

The differences in liver cell mortality after administration of mangosteen peel extract in rats after induction with formalin showed that there were significant differences in mean numbers of liver cell deaths between the three intervention groups. The group receiving mangosteen peel extract at a dose of 400 mg/ kgBW/day for one week, after a 3-week administration of formalin, showed the lowest mean liver cell mortality rate at 610.67 ± 59.742 cells (p=0.004). The differences in results after post-hoc Tukey HSD anlysis are presented in Table 1.

In the control group given only formalin, the cells had a high mortality in comparison with the treatment groups 1 and 2. The histopathological picture of the control group shows a number of liver cells with pycnotic nuclei, while some of the cells had karyorhexis and karyolysis (Figure 2A). In the treatment groups, however, a number of liver cells still had intact nuclei (Figures 2B and 2C).

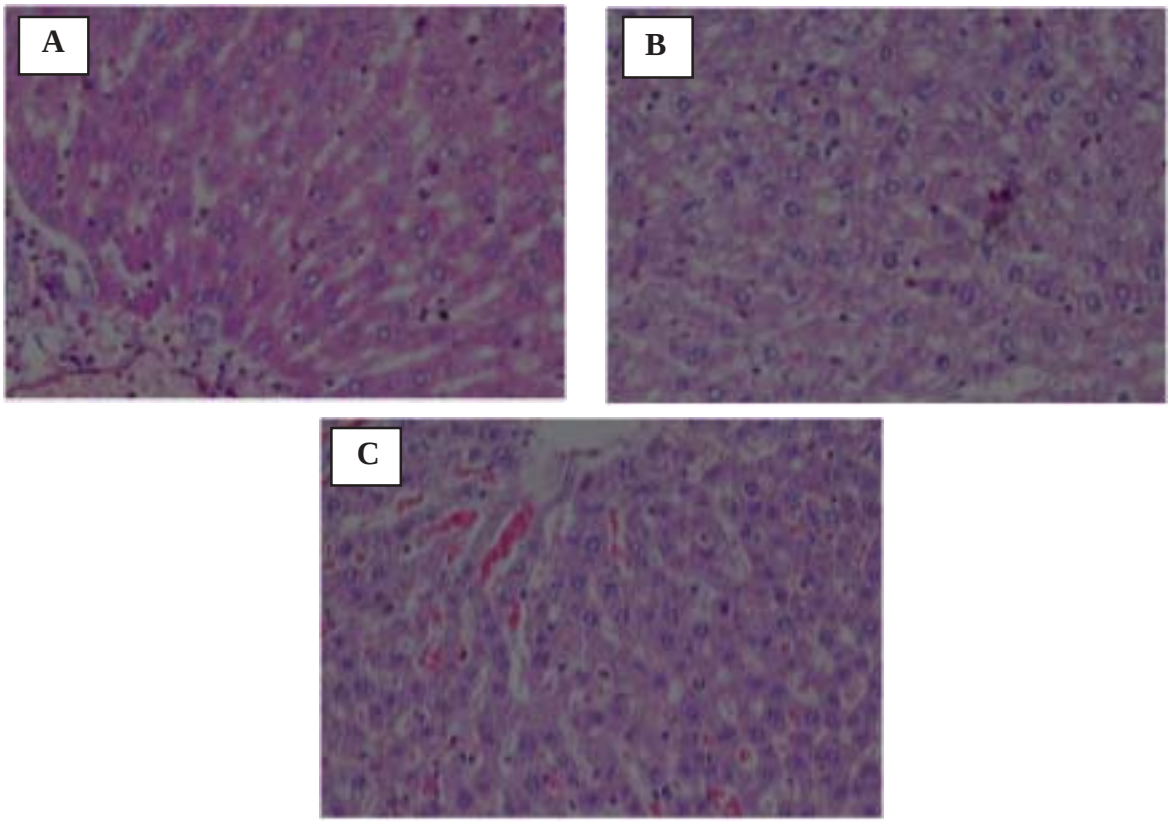

Figure 2 . Histopathological examination of the three treatment groups, viz. control group(A), treatment 1 (B) and treatment 2 (C) (magnification 400x)

Between the formalin-control group and treatment group 1 (mangosteen peel extract 200 mg/kgBW) there were no significant differences in the number of dead liver cells (p=0.398), showing that mangosteen peel extract at a dose of 200 mg/kgBW had no appreciable effect on cellular recovery from formalin lesions. However, between the control group and treatment group 2 (mangosteen peel extract 400 mg/kgBW) a significant difference was found (p=0.004), indicating that mangosteen peel extract at a dose of 400 mg/kg BB induced cellular recovery from formalin lesions.

DISCUSSION

From the results of liver cell mortality rate assessment in our study, it is apparent that mangosteen peel extract was capable of effecting repair of histological liver cell structures that had been exposed to formalin. These observations are in agreement with those of previous studies. The study by Pedraza-Chaverri et al.[12] showed that xanthones isolated from mangosteen peel had pharmacologic effects, such as antioxidant and anti-inflammatory effects. The xanthones gamma-mangostin and alpha-mangostin contained in mangosteen peel showed radical scavenging activity.[17] Gamma-mangostin also had anti-inflammatory effects through inhibition of cyclooxygenase (COX) activity. The study conducted by Kosem et al.[10] showed that methanolic mangosteen peel extract had antioxidant and cytoprotective effects on endothelial cells.

Cell death from formalin is a chemical lesion capable of causing cellular damage and death. Swelling of the cell is the first morphological change to be seen, and is reversible in nearly all forms of cellular lesion. In advanced stages due to progressive lesions, the cell passes the point of no return, and enters the irreversible stage of cell death. The signs of cell death are to be seen in the cell nucleus. The earliest nuclear change is karyolysis, with the basophilic chromatin becoming pale, which is due to activation of DNAse from decreased cellular pH. The second change is pycnosis, with shrinkage of the nucleus and increased basophilic staining, where the DNA condenses into a solid, basophilic, shrunken mass. The third possible change is karyorhexis, with pycnotic nucleus, or fragmentation and disappearance of the pycnotic nucleus.[18-20]

Reversible cellular lesions may be decreased by the antoxidant and anti-inflammatory effects of mangosteen peel, and do not progress to the stage of irreversibility. Liver cells that are still in the stage of reversible damage may recover, so as to minimize cell death or irreversible cellular damage.[20]

The results of histopathological examination showed that p53 protein expression was negative in all groups (Figures 1A, B, and C). There have been many studies conducted on the process of cell death or cellular damage using p53 expression as indicator, such as in mammary adenocarcinoma. The studies in question showed increased p53 expression after a 3-week administration of ant-nest extract to mice with mammary adenocarcinoma. This indicates that p53 is a trigger protein for apoptosis of mammary cancer cells.[15] Expression of p53 is also seen in cisplatin-induced cells.[15]

In our study there were no cells expressing p53 protein. This may have been caused by the lack of opportunity of the cellular death pathway due to formalin-induced chemical lesions to relay instructions for p53 activation. Formalin lesions are traumatic chemical lesions causing cell damage and necrotic death resulting from ROS production and histological hypoxia. Cell death from formalin lesions is necrotic death without p53 involvement. The cell death in the present study was acute, being a traumatic chemical lesion directly affecting morphological cell structures. The cells in our study were normal cells without carcinogenic initiation, mutation, or chronic processes.

The observations in our study were short term, whereas cellular dynamics are ongoing processes. This constitutes a limitation of our

study, since it is possible that during the short observation period, there had been no opportunity for expression of p53 proteins leading to cell death. Further studies are needed to confirm the involvement of apoptosis in cell death due to oral formalin exposure. With regard to clinical application, the present study is expected to become the foundation study for discovery of antioxidant herbal drugs, to be utilized in humans for the cure or prevention of free radical-induced tissue damage.

CONCLUSIONS

Mangosteen peel extract effects celullar recovery from formalin-induced chemical lesions. Involvement of p53 expression in liver cell mortality rate in rats exposed to oral formalin is presumably negligible.

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