

Effect of *Berberis vulgaris* Aqueous Extract on the Apoptosis, Sodium and Potassium in Hepatocarcinogenic Rats

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Abstract

Objective(s)

The effect of *Berberis vulgaris* aqueous extract in hepatocarcinogenic rats was studied to investigate the apoptotic and sodium, potassium elements properties. A loss of both intracellular potassium and sodium occurs when apoptotic cells shrink and prior to the loss of membrane integrity.

Materials and Methods

Forty-eight *Sprague dawley* rats were randomly divided into 2 groups, normal and cancerous. Each group was divided into 4 subgroups. The first subgroup acted as normal control while the others were treated with 25, 50 and 100 mg/kg of *Berberis vulgaris* extract (BVE) and respectively considered as NC, NC25, NC50 and NC100. The first subgroup of cancerous rats acted as cancer control while the others were treated with 25, 50 and 100 mg/kg of BVE and considered as C, C25, C50 and C100. Ion selective electrode (ISE) method was used to measure the level of sodium, potassium, and chloride. TUNEL assay used for the detection of apoptosis cells.

Results

Microscopic observations of the TUNEL-positive apoptotic cells revealed a significant difference ($P < 0.05$) between cancer control (C) and normal control (NC) group. The results indicated that increasing concentration of *Berberis vulgaris* aqueous extract in cancerous treated groups (C25, C50 and C100) showed an increasing considerable changes ($P < 0.05$) of TUNEL-positive cells compared with the cancer control group (C). Sodium and chloride levels were significantly different ($P < 0.05$) in cancer control group (C) compared to normal control group (NC). The results suggested that apoptotic cells level was increased with the BVE concentration in cancerous groups.

Conclusion

The *Berberis vulgaris* extract shows to play a prominent role in promoting apoptosis on the treatment and it is dose dependent.

Keywords: Apoptosis, *Berberis vulgaris*, Elements, Hepatocarcinogenesis

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Introduction

Cancer has become an important topic in medicine since it is a major cause of death in both the developed and developing countries (1). Liver cancer is the fourth leading cause of cancer mortality worldwide and results in more than 400,000 deaths annually (2). The geographical distribution of liver cancer is very uneven; 83% of cases occur in developing countries (3). Treatment of cancer is a multidisciplinary effort (4). Most anticancer agents now in use were developed using empirical screens design to identify agents that selectively kill tumor cells (5).

Berberis vulgaris have long been used as a herbal remedy for the treatment of a variety of complaints. It is one of the medicinal plants that have received great interests among researchers and most significant European representative of the Berberidaceae (6). Barberry grows in Asia and Europe and the plant is well known in Iran (7). All parts of the plant can be used (8) and it is mainly taken nowadays as a tonic to the gallbladder to improve the flow of bile and ameliorate conditions such as gallbladder pain, gallstones and jaundice (9). The fruit, or freshly pressed juice, is used in the treatment of liver and gall bladder problems, kidney stones, menstrual pains etc (10). Barberry contains a wealth of anticancer, antitumor, antioxidant, and mutation-preventing compounds, as well as cancer-preventive properties (11). Barberry has been used for cancer or tumors of the liver (12).

The role of elements in cancer is not well understood, although some are known to be carcinogenic while others seem to have a protective effect against carcinogenesis. Antioxidant element is any substance that hinders a free radical reaction (13) or a substance capable of neutralizing oxygen free radicals. Changes in the level of elements in cancer tissues have been demonstrated in many previous studies (14). The changes in concentrations of a number of elements are determined by neutron activation analysis in tumor, liver, and blood serum of host animals, following local irradiation of a solid tumor (15, 16). The cell shrinkage is a ubiquitous

characteristic of programmed cell death that is observed in all examples of apoptosis, independent of the death stimulus. This decrease in cell volume occurs in synchrony with other classical features of apoptosis. The molecular basis for cell shrinkage during apoptosis involves fluxes of intracellular ions including K, Na, and Cl (17).

In this study, the identification of apoptotic on liver tissue and the level of K, Na, and Cl elements were studied on hepatocarcinogenic rats treated with three different concentrations of aqueous *Berberis vulgaris* extract. The significance and the outcome of this study could be associated with prognosis, which are very important in a medical treatment of cancer.

Materials and Methods

Preparation of Berberis vulgaris extract

Berberis vulgaris fruit extract (BFE) prepared by method of Shamsa *et al* (7). *Berberis vulgaris* that was free from fungus, bacteria and any other plant diseases selected randomly. The fruits were dried in an oven for 3 days at a constant temperature of 65 °C. The fruits cut into small pieces and grounded into fine powder using a dry grinder. The grounded samples sieved to get uniform particle size, then kept in air-tight container and stored for further extraction. Water and ethanol added in the ratio of 1:10 and stirred at 250 rpm in an orbital shaker for 1 hr at room temperature. The extract was then separated from the residue by filtration through Whatman No.1 filter paper. The remaining residue re-extracted twice, and then the two extracts were combined. The residual solvent of ethanolic extract removed under reduced pressure at 50 °C, using a rotary evaporator until thick syrup was collected. The thick syrup evaporated completely, using freeze drying system.

Animal treatment

Forty-eight *Sprague dawley* rats (150-200 g weight, 6-8 weeks old) purchased from animal colony unit, Faculty of Medicine, University of Putra, Malaysia. They were randomly divided into 2 groups, normal and cancerous.

Each group was divided into 4 subgroups and the first group of 4 subgroups acts as normal control (NC) and cancer control (C) while the others were treated with 25, 50 and 100 mg/kg of *Berberis vulgaris* extract. Hepatocarcinogenesis was induced according to the method described by Solt and Farber (1976) (18) but without partial hepatectomy. The hepatocarcinogen diethylnitrosamine (DEN; Sigma) was injected intraperitoneally at a dose of 200 mg/kg of body weight and after 2 weeks, the rats were fed with a diet containing 0.02% (w/w) 2-acetylaminoflourene (AAF; Sigma) for another 2 weeks. Of *Berberis vulgaris* extract, 1.0 ml according to certain concentration (25, 50 and 100 mg/kg) were given through force-feed to the normal and cancerous treated groups (NC25, NC50, NC100, C25, C50 and C100) until the end of experiment.

All animals were sacrificed at week eleven. The blood was collected from the heart into heparinized tubes and then centrifuged (Universal 30RF, Hettich Zentrifuge, Tutlingen). The serum samples used for elements evaluation. The livers removed, weighed and cut transversally. Liver slices fixed in 10% formalin, processed and sectioned.

Detection of apoptotic cells

Terminal deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) assay (DeadEnd™ Fluorometric TUNEL System; Promega) was performed according to the manufacturer's instruction. The 4 µm thick tissue sections were first deparaffinized, rehydrated through a graded alcohol series and washed in 0.85% NaCl and PBS. After 15 mins of fixing in 4% methanol-free formaldehyde solution and 5 mins of washing in PBS, 100 µl of the 20 µg/ml proteinase K was added to each slide and incubated for 8-10 mins at room temperature. The cells were covered with 100 µl of Equilibration Buffer and equilibrated at room temperature for 5-10 mins. Terminal deoxynucleotidyl transferase (TdT) incubation buffer 50 µl was

added to the cells on a 5 cm² area, covered with plastic coverslips and incubated in a humidified chamber for 60 mins at 37 °C. The reaction was terminated in 15 mins at room temperature and washed in fresh PBS. The samples were stained in 40 ml of propidium iodide solution that freshly diluted to 1µg/ml in PBS for 15 mins at room temperature in the dark. Finally, after washing and draining the excess water from the slides, the samples were analyzed under the fluorescence microscope. All the slides examined and a systematic scoring method was done at ×20 magnification and 5 spots were selected. On each spot, green stained cells counted as TUNEL-positive apoptosis cells.

Elements analysis

Serum was stored at freezer -70 °C and elements were analyzed by using a fully automated analyzer Hitachi 902. Ion selective electrode (ISE) is a method that was used to measure the level of sodium, potassium, and chloride.

Statistical analysis

The results were analyzed using one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant. The statistical package for social science, for windows version 11.5 was used to analyze the data.

Results

Table 1 shows mean of the body weight increment, liver weight and relative liver weight of all groups. Overall, body and liver weight of all hepatocarcinogen-induced rats (C, C25, C50 and C100) did not show any significant difference ($P > 0.05$) in comparison with the normal control and normal with treated groups (NC, NC25, NC50 and NC100). All normal and cancerous treated groups (NC25, NC50, NC100, C25, C50 and C100) did not show any significant changes of body and liver weight, compared to the normal control group (N).

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Table 1: Effect of *Berberis vulgaris* extract on body and liver weight after 11 weeks of treatment. Values shown are mean \pm SEM Mean with the same superscript are not significant at $P<0.05$.

Group	Body Weight Increment Mean \pm SEM (%)	Liver Weight Mean \pm SEM (g)	Relative Liver Weight Mean \pm SEM (g)
NC	24.14 ^a \pm 2.912	6.54 ^a \pm 0.199	0.000284 ^{a,b} \pm (8.65 \times 10 ⁻⁶)
NC25	27.45 ^a \pm 5.537	6.32 ^a \pm 0.105	0.000282 ^{a,b} \pm (6.81 \times 10 ⁻⁶)
NC50	21.41 ^a \pm 3.711	6.08 ^a \pm 0.302	0.000276 ^{a,b} \pm (1.98 \times 10 ⁻⁵)
NC100	19.59 ^a \pm 3.519	5.89 ^a \pm 0.164	0.000258 ^a \pm (7.54 \times 10 ⁻⁶)
C	15.40 ^a \pm 5.850	6.56 ^a \pm 0.451	0.000312 ^b \pm (1.64 \times 10 ⁻⁵)
C25	18.05 ^a \pm 8.483	6.18 ^a \pm 0.244	0.000308 ^b \pm (1.29 \times 10 ⁻⁵)
C50	22.45 ^a \pm 5.617	6.40 ^a \pm 0.193	0.000285 ^{a,b} \pm (8.06 \times 10 ⁻⁶)
C100	12.86 ^a \pm 6.799	6.01 ^a \pm 0.675	0.000269 ^{a,b} \pm (2.11 \times 10 ⁻⁵)

NC= normal control, NC25= normal + 25 mg/kg BVE, NC50= normal + 50 mg/kg BVE, NC100= normal + 100 mg/kg BVE, C= cancer control, C25= cancer + 25 mg/kg BVE, C50= cancer + 50 mg/kg BE, C100= cancer + 100 mg/kg BVE.

Cancer control (C) rats showed a significant ($P<0.05$) more increase in the number of TUNEL-positive cells (apoptotic cells number) than the normal control (NC) rats. TUNEL-positive cells apoptotic count was significantly high ($P<0.05$) in all cancer treated groups compared to the normal control (NC) rats. TUNEL-positive apoptotic cells increased

significantly ($P<0.05$) in cancer treated with 50 and 100 mg/kg group (C50 and C100) compared to cancer control group (C). There was significant ($P<0.05$) difference in the normal group treated with 50 mg/kg (NC50) compared to the normal control group (NC) (Figure 1).

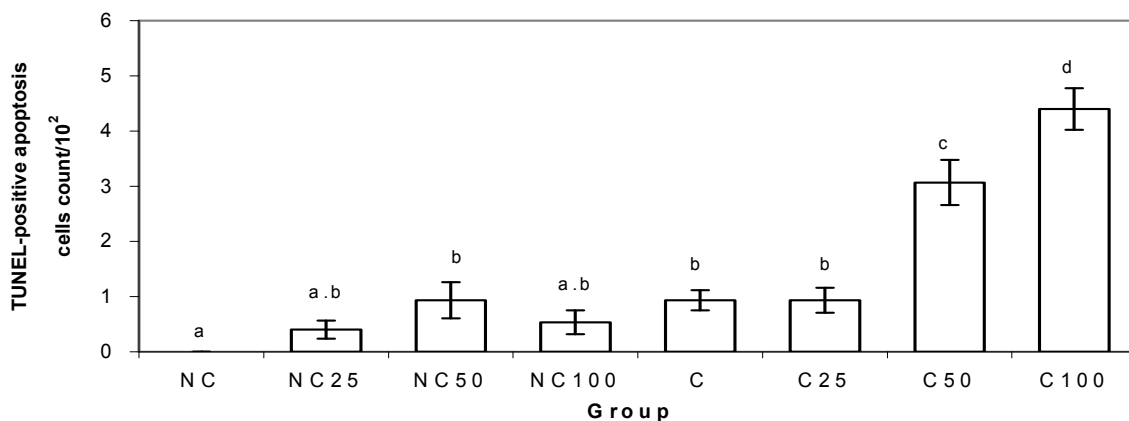


Figure 1. Quantification of TUNEL-positive cells in liver sections of NC = normal control, NC25 = normal + 25 mg/kg BVE, NC50 = normal + 50 mg/kg BVE, NC100 = normal + 100 mg/kg BVE, C= cancer control, C25= cancer + 25 mg/kg BVE, C50= cancer + 50 mg/kg BE, C100= cancer + 100 mg/kg BVE. Data with the same alphabet are not significant at $P<0.05$.

Table 2 summarizes sodium, potassium and chloride levels in rat serum. Both C25 and C100 groups had an equal and the highest potassium level compared to other groups. High sodium and chloride levels can be seen in the cancer control, that differ significantly ($P<0.05$) with the normal control rats. Sodium and chloride levels of all normals treated with *Berberis vulgaris* extract did not show any significant difference when compared to the

normal control rats. There was increase in sodium and chloride levels, in all cancerous treated groups compared to the cancer control group, however it was not significant. There was no significant difference in the potassium level of cancer control as compared with the normal control rats. Potassium level of all non-cancerous treated rats did not differ significantly, in comparison with the normal control group.

Table 2. The mean antioxidant elements level after 11 weeks of treatment. Values shown are mean±SEM Mean with the same superscript are not significant at $P<0.05$.

Group	Sodium Level Mean±SEM (mmol/l)	Potassium Level Mean±SEM (mmol/l)	Chloride Level Mean±SEM (mmol/l)
NC	201.67 ^a ±6.844	7.33 ^a ±0.543	148.67 ^a ±5.584
NC25	194.67 ^a ±7.597	7.60 ^{a,b} ±0.531	144.33 ^a ±5.993
NC50	216.83 ^{a,b} ±7.952	7.20 ^a ±0.649	161.00 ^a ±5.398
NC100	199.00 ^a ±16.675	7.37 ^a ±0.964	146.17 ^a ±13.085
C	257.60 ^c ±20.314	9.62 ^{a,b} ±1.071	194.6 ^c ±15.964
C25	254.20 ^c ±19.986	10.56 ^c ±1.103	192.60 ^c ±15.390
C50	223.20 ^{a,b} ± 6.119	9.06 ^{a,b} ±1.500	170.20 ^{a,b} ±5.113
C100	229.20 ^{a,b} ±10.566	10.56 ^c ±1.246	173.60 ^{a,b} ±9.003

NC = normal control, NC25 = normal + 25 mg/kg BVE, NC50 = normal + 50 mg/kg BVE, NC100 = normal + 100 mg/kg BVE, C = cancer control, C25 = cancer + 25 mg/kg BVE, C50 = cancer + 50 mg/kg BE, C100 = cancer + 100 mg/kg BVE.

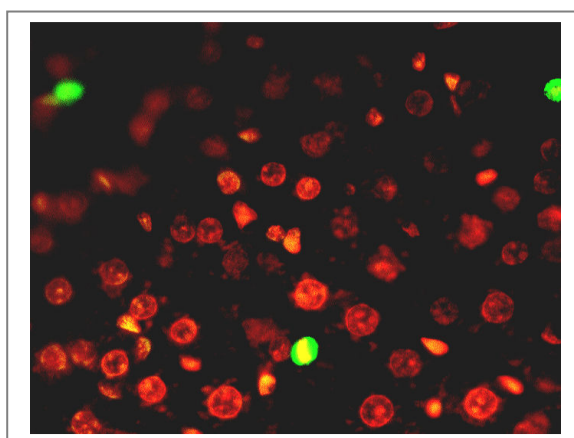


Figure 2. DNA Fragmentation detected by TUNEL assay in apoptosis cells. Fluorescence micrograph showed green fluorescent labeled nuclei as TUNEL-positive apoptosis cells in cancer group treated with 100 mg/kg BVE (C100) at ×400.

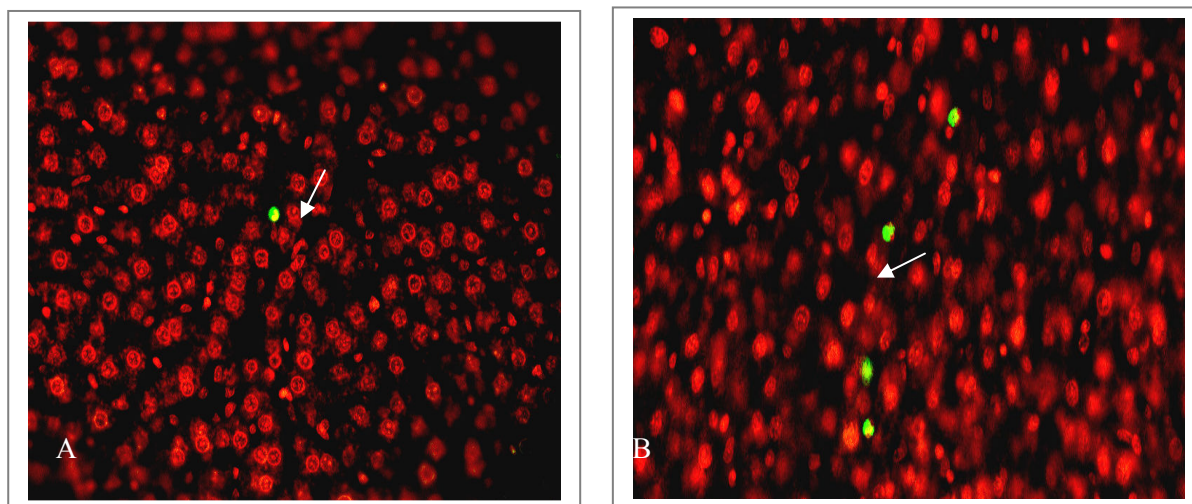


Figure 3. DNA fragmentation detected by TUNEL assay in apoptotic cells. Fluorescence micrographs showing green fluorescent labeled nuclei (arrow) as TUNEL-positive apoptosis cells in NC50 (A) and C50 (B) at ×200.

Discussion

The decrease of body weight observed in hepatoma is a symptom common in malignant tumours (19) and it is very much associated with the severity of neoplasia (20). It is

believed that cancer causes loss of appetite in both animals as well as in human, thus reducing the food intake in cancer rats (21). Proliferation of cells will increase the size of the liver (22). However, in this study, the results of body weight, liver weight and

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relative liver weight after eleven weeks of bioassay did not show any significant difference between NC and C groups. Overall, body weight increment profile of all hepatocarcinogen-induced rats did not show any significant difference, compared to the normal control. Body weight profile of the rats might also be affected by other factors such as age, environment, genetic, health level and diet changes associated with food consumption (23). Supplementation of BVE did not influence the body and liver weight of normal rats indicating that treatment of BVE to normal rats did not alter the body increment or food intake progress of the rats.

Apoptosis is characterized by a series of well-documented morphological changes, which can be observed by light and electron microscopy. Within the nucleus, compaction of nuclear chromatin occurs, which then becomes margined against the nuclear envelope and subsequently there is nuclear fragmentation (24). TUNEL procedures rely on labeling of breaks in the DNA strands that have been observed to occur in apoptosis (25). Quantitative determination of apoptosis in histological specimens by Schulte-Hermann *et al* (1995) revealed (26) that the rate of apoptosis tends to increase from normal to (pre) neoplastic to malignant cells (27). The result showed that there were significant difference ($P < 0.05$) of TUNEL-positive apoptosis cells count in the cancer control group, compared to the normal control group.

It is now established that anticancer agents induce apoptosis and that disruption of apoptotic programs can reduce treatment sensitivity (28). Surprisingly, the result of authors other study indicated that high dose of *Berberis vulgaris* extract has an anticancer effect towards DEN-initiated and AAF-promoted hepatocarcinogenesis in rats (12). It is possible that tumors which exhibit apoptosis may be more sensitive to chemotherapy and hence like to have a better prognosis (28). Thus, tumours which exhibit more apoptosis may be slower growing and, therefore, less biologically aggressive (24).

Supplementation of 50 mg/kg *Berberis vulgaris* extract to normal rats significantly affected the apoptotic cells count, compared to normal control group. Anticancer agents induce apoptosis in normal tissues as well as in tumors (29). In fact, many of pathologists who identified apoptosis in tumors realized that apoptotic cells death was induced in a subset of normal tissues (e.g. bone marrow and intestine) and it was suggested that the process might contribute to the 'toxicity' associated with chemotherapy (30). However, toxicity cannot be provide associated with the supplementation of the extract because there was no significant difference in the TUNEL-positive apoptotic cells count observed in non-cancerous group treated with 100 mg/kg *Berberis vulgaris* extract compared to the normal control group.

The observation of sodium and chloride level in the cancer control compared to normal control suggests that a clinical monitoring of elements in serum may be used to predict chemotherapeutic efficacy (31). The major distinction between the cancer and normal control appears to be the increased extracellular water compartment of the cancer group (32). The changes in level of sodium in cancer compared to the normal control may also, be explained by a breakdown in the active transport of the ions through the cell membranes.

In this study, we showed that an early increase in intracellular sodium, potassium and chloride induced by a variety of apoptotic cells. However, surprisingly, the results were not significant. This could be due to the duration of study.

Conclusion

Distinct evidences from this study contribute to the new finding towards anticancer agent from a plant fruits, *Berberis vulgaris* contributing to the effort of treatment and suppression of the liver cancer diseases. The *Berberis vulgaris* extract play a prominent role in promoting apoptosis upon the treatment and it is dose dependent.

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