In vitro evaluation of Rheum ribes induced genotoxicity in HepG2 cell lines

Mahmoud Abudayyak
Department of Pharmaceutical Toxicology, Karadeniz Technical University, Faculty of Pharmacy, Trabzon, Turkey

ABSTRACT
Rheum ribes is a perennial herbaceous plant belonging to the Polygonaceae family that grows more on rocky and gravelly slopes in high altitude areas of Levant and Turkey. Rheum ribes is consumed as food and widely used in folk medicine against nausea, constipation and for different diseases including diabetes and hypertension. Unfortunately, the research on Rheum ribes toxicity is insufficient. In our study, the human hepatocellular carcinoma (HepG2) cell line was used in a cytotoxicity evaluation of Rheum ribes water, methanol and chloroform extracts by MTT and NRU tests. Comet assay was used to investigate the genotoxicity potentials of the plant extracts. Our results show that all extracts cause cell death in a concentration dependent manner at 5-50 mg/mL concentrations. The IC50 values are 14.29-31.94 mg/mL by MTT and 21.15-27.66 mg/mL by NRU assay. The highest concentration (25 mg/mL) of methanol extract causes significant DNA damage (8.7-folds). In conclusion, similar to a lot of plants used in folk medicine the risk of Rheum ribes is still unknown. The uncontrolled use of this plant could cause harm to the patients. Our results indicate the possible cytotoxic and genotoxic effects of Rheum ribes, these results should elevate concerns about the safety of Rheum ribes and other folk herbs.

Keywords: Rheum ribes, herbal toxicity, genotoxicity, cytotoxicity, HepG2 cells

INTRODUCTION
Mankind has been discovering the therapeutic power of plants and benefiting from the herbal power to survive and fight diseases since ancient times. In developing countries, more than 80% of the population still use traditional medical plants as the first choice in the treatment of different diseases. About 80% of the world’s population is thought to be living in developing countries; which means that about 64% of the world’s population uses herbal remedies (Farnsworth et al., 1990). In addition to this, approximately 25% of the drugs sold by prescription in developed countries are herb-derived chemicals (Principi et al., 1991). In developed countries a new wave of “back to nature” has affected individuals and communities leading to an increased interest in alternative medicine causing increases in the use of herbal medicines. Additionally, the high costs of pharmaceutical and health protection products are pushing a large part of the population of developing countries towards choosing traditional remedies in the treatment of their disease (Verschaeye et al., 2004).

Rheum ribes L. (known in Turkish as Ribês, Réwas, Reweş, Uçkun, Işkın, Işgın) is a perennial herbaceous plant belonging to the Polygonaceae family (Öztürk et al. 2007; Korkmaz et al. 2015; Polat et al. 2015). It is located in Palestine, Lebanon, Armenia, Iraq, Iran and the Eastern regions of Turkey (Ağrı, Bingöl, Elaziğ, Hakkari, Kars, Van and Sivas), and mainly grown on the rocky and gravelly slopes at high altitude areas (Otoom et al. 2006; Öztürk et al. 2007; Cakilcioglu et al. 2010; Polat et al. 2013).

Rheum ribes is consumed fresh, and cooked as a jam (Cakiliçoglu et al. 2011; Polat et al. 2015). Also, it is assumed to be a very important herb with different uses in the folk medicine of Turkey and Iran. The root and fruit (stem part) in particular, are frequently
used in diabetes, high blood pressure, cholesterol, cirrhosis, arthritis, Alzheimer and other diseases (Abu-Irmaileh et al. 2003; Otoom et al. 2006; Nasirshban et al. 2009; Sayyah et al. 2009; Kasabri et al. 2011; Polat et al. 2013; Hamzeh et al. 2014; Zahedi et al. 2015). Also, it is widely consumed to combat nausea and constipation (Oktay et al. 2007; Tetik et al. 2013).

Although the therapeutic, pharmacognostic, antibiotic and some of biochemical characteristics of *Rheum ribes* have been well studied, the toxic potential studies are very few and insufficient. For this reason, in this study the cytotoxic and genotoxic potential of methanol, chloroform and water root extracts were evaluated in HepG2 human hepatocarcinoma cells used previously as models of *in vitro* conditions to study the apical uptake, metabolism and absorption of nutrients and the toxicity of chemicals and drugs (Martin et al. 1997; Brand et al. 2000; Goya et al. 2015).

**MATERIALS AND METHODS**

**Materials**

Human hepatocarcinoma HepG2 cell line (HB-8065) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell culture medium (Eagle's minimum essential medium, EMEM), Phosphate buffer solution, Fetal bovine serum (FBS), Trypsin-EDTA solution and the antibiotic solution (100 IU/mL penicillin and 100 mg/mL streptomycin) were obtained from Wisent Bioproducts (Montreal, Canada), and all the other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Dried roots of *Rheum ribes* (*Rizoma Rhei ribi*) were purchased from herbalists and spice sellers in Istanbul. Plant samples were tested and identified by Prof. Dr. Emine Akalin (Istanbul University, Department of Pharmaceutical Botany).

**Plant extractions**

Three different extracts of *Rheum ribes* were prepared using water, methanol and chloroform. For this, the roots were pulverized, 2.5g of this powder was then treated with 25 mL methanol or chloroform for 30 minutes in a water bath shaker at 25°C. A rotary evaporator and steam from nitrogen gas (40°C) were used to concentrate and dry the extracts. After dissolving the solid residue in 1 mL of dimethyl sulfoxide (DMSO), the solutions were filtrated using 0.45 µm filters. For the water extract, the infusion method was performed. 25 mL of hot (90°C) sterile water was added to 2.5 g of root powder and stirred for 30 minutes at a fixed temperature (90°C). After cooling, the mixture was filtered with filter paper and 0.45 µm filters. The methanol and chloroform extracts concentrations were 2500 mg/mL, the water extract concentration was 100 mg/mL (Abudayyak et al. 2015).

**Cell culture and exposure**

Human hepatocarcinoma cells (HepG2) were cultured in EMEM medium supplied with 10% heat inactivated FBS and 1% antibiotics. The cells were incubated at 37°C, 90% humidity and 5% CO₂ (confluence 60-80%). 96-well plates were used for the cytotoxicity assays and 6-well plates for the genotoxicity assay. The cell density was 1x10⁴-5x10⁴ cell/ well for the cytotoxicity assays and 1x10⁶ cell/ well for the genotoxicity assay. The final exposure concentrations were 0.25-50 mg/mL, the exposure period was 24 hours.

**Cytotoxicity evaluation**

The cytotoxic effects of *Rheum ribes* root extracts were evaluated using MTT and NRU assays. After the incubation with different extract concentrations (5-50 mg/mL), the exposed cells were treated with MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) for 2 hours, the succinate dehydrogenase enzyme in the viable cells metabolized the yellowish water soluble MTT to water non-soluble formazan violet crystals. In the NRU assay, the weak red cationic dye accumulates in integral and healthy cells by creating electrostatic hydrophobic bonds with the lysosomal matrix. The cellular enzyme activity in the MTT assay and the integrity of the cells in the NRU assay are evaluated as a sign of cells' viability. A Microplate spectrophotometer system (Epoch, Germany) at 540 nm and 590 nm was used to measure the optical densities (ODs) for NRU and MTT, respectively. The nonexposed cells and cells exposed to DMSO (1%) were evaluated as negative and solvent controls respectively. The concentration – cell death (%) curves were used to calculate the median inhibitory concentrations (IC₅₀) that were responsible for the death of 50% of the cells (Mosmann 1983; Borenfreund et al. 1985).

**Genotoxicity evaluation**

The Comet assay was used to evaluate the genotoxic potential of *Rheum ribes* rhizomes extracts in the HepG2 cells. The concentrations of exposure were 6.25; 12.5 and 25 mg/mL for both methanol and chloroform extracts and 0.25; 0.5 and 1 mg/mL for water extract. For this, the exposed cells were trypsinized, washed with PBS 1X and mixed with pre-warmed low-melting point agarose. Cells were layered on agarose pre-coated microscope slides, covered with a cover slip and allow to solidify. After fixation on the slides, the cells were treated with lysis solution for one night. The slides were washed and incubated for 20 min in fresh a cold electrophoresis buffer before electrophoresis for 20 min, and treated with a neutralization buffer for 15 minutes. Before evaluation under a fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) the cells were stained with ethidium bromide. The Comet analysis and scoring program (Comet Assay IV, Perceptive Instruments, Suffolk, UK) was used to image and score at least one hundred cells per sample. DNA damage to individual cells was expressed as a percentage of DNA in the comet tail (mean tail intensity %) (Singh et al. 1988; Abudayyak et al. 2017). The nonexposed cells and cells exposed to DMSO (1%) were evaluated as negative and solvent controls respectively. For genotoxicity evaluation hydrogen peroxide (H₂O₂; 100 µmol/L) was used as a positive control.

**Statistical analysis**

Cytotoxicity assays were done in triplicate, experiments were also repeated four times on different days (n=12). The genotoxicity evaluation was done in triplicate. Data is expressed as mean ± standard deviation (SD). The significance of the differences between negative control and exposed cells was evaluated using one-way analysis of variance (ANOVA) and Dunnett t-test by The Statistical Package for the Social Sciences (SPSS) version 23.0 for Windows (IBM Corp.; Armonk, NY, USA). P values of less than 0.05 were selected as the levels of significance.
RESULTS AND DISCUSSION

*Rheum ribes* is used as a raw and cooked food in Middle East culture (Oktay et al. 2007; Özcan et al. 2007), it is also widely used as a medical herb (Abu-Irmaileh et al. 2003; Alaadin et al. 2007; Özcan et al. 2007; Naqishbandi et al. 2009). The research related to *Rheum ribes* has mainly focused on the ethnopharmacological relevance (Afifi et al. 2000; Abu-Irmaileh et al. 2003; Cakilcioglu et al. 2010; Najafi et al. 2012; Nabati et al. 2013; 2015; Kaval et al. 2015; Korkmaz et al. 2015), pharmacognostic characterization (Munzuroglu et al. 2000; Tosun et al. 2003; Özcan et al. 2007; Andić et al. 2009; Naemi et al. 2014; Amiri et al. 2015), therapeutic (Otoom et al. 2006; Gholamhosseini et al. 2009; Naqishbandi et al. 2009; Sayyah et al. 2009; Sindhu et al. 2010; Zehedi et al. 2015) and antioxidant (Öztürk et al. 2007; Krishnaiah et al. 2011) effects of *Rheum ribes*. There are only very few works related to the toxic potential of the plant. Sardari et al. (2009) evaluated the cytotoxic effect of ethanol extracts of the herb *Rheum ribes* in different cell lines by MTT test, with the results showing IC<sub>50</sub> values ranging between 11.2-67.96 mg/mL. Esmaeilbeig et al. (2015) evaluated the anti-cancer effect of different *Rheum ribes* extracts against tumor cells using MTT cytotoxicity assay – the results showed that IC<sub>50</sub> was 115 µg/mL in human blood (K562) cell line while 200 µg/mL concentration caused less than a 15% decrease in the viability of Hela cells. Similarly, Cinar et al. (2016) calculated the IC<sub>50</sub> to be 400 µg/mL in MCF-7 breast cancer cells. An in vivo study evaluated the acute and sub chronic toxicity (for 60 days) of *Rheum ribes* aromatic water in the Wistar rat, an increases of some enzymes like lactate dehydrogenase, abnormality in heart with tissue hemorrhage, hypertrophy and infiltration of inflammatory cells were noticed, the not observed adverse effect level (NOAEL) was calculated to be 250 and 500 mg/kg b.w/day for male and female rats, respectively (Mojarrab et al. 2015).

In this work exposure to *Rheum ribes* extracts for 24 hours caused a decrease in the viability of HepG2 cells dependent on concentration manner. The MTT assay results show that IC<sub>50</sub>
values are 14.29; 33.67; and 31.94 mg/mL for the extracts of water, chloroform and methanol, respectively (Figure 1). The IC_{50} values for the NRU assay for water and methanol were 21.15 and 27.66 mg/mL, respectively (Figure 2). At the highest concentration (50 mg/mL) of chloroform extract, cellular death was 32.8%. The differences between the cytotoxicity assays were discussed previously and the different results obtained in these different assays were reported (Weyermann et al. 2005; Fotakis & Timbrell 2006). There were many factors including the interaction between the tested xenobiotics and the chemicals of the assay (Wang et al. 2010). In the case of NRU some chemicals decrease the lysosomes account in the cells, leading to negative false results. Additionally, some chemicals may increase the activity of succinate dehydrogenase enzymes causing false positive results in the MTT test. Similarly, chemicals affecting cellular adherence could also cause cells loss leading to false positive results. This could explain the difference in chloroform extracts between MTT and NRU assays.

To the best of our knowledge the genotoxicity of Rheum ribes has not been evaluated previously. Comet assay results show that Rheum ribes water and chloroform and the low concentrations of methanol extracts did not cause any significant DNA damages after 24 hours exposure. Only the highest concentration of methanol extract (50 mg/mL) causes significant DNA damage (8.7-folds) in HepG2 cells (Figure 3).

Unfortunately, there is very little research which discusses the difference in toxicity between different herbal extraction methods. According to this research, it is also difficult to argue that methanol extracts are more toxic than chloroform ones or vice versa. In a previous study, the toxicity of the extracts of ten herbs was evaluated with the MTT test. The results showed that the chloroform extracts of all the test herbs were more cytotoxic than the methanol extracts and none of the water extracts showed any cytotoxicity. The chloroform extracts (in general) possessed more mutagenic activity with the Ames test than methanol and water extracts (Abudayyak et al. 2015). Similar results were also found by Chan et al. (2015). In contrast, previous studies showed that the chloroform and water extracts of Tribulus terrestris were less cyto- and genotoxic than methanol extracts (Abudayyak et al. 2015 B). However, in this study, the results indicate that while the methanol extracts were less cytotoxic than the water and chloroform extracts, they possessed the highest genotoxicity.

**CONCLUSION**

Contrary to the popular belief that herbs are safe because they are natural products, some herbs can cause significant toxic effects, drug interactions, and even morbidity or mortality. It would be beneficial to evaluate at the very least the cytotoxicity, genotoxicity and carcinogenicity of these herbs in order to assess the associated risks to our health. *Rheum ribes* is one of herbs that are consumed frequently in Turkey and Middle East countries yet the data concerning the safety of *Rheum ribes* is still insufficient. There is a need for *in vivo* and *in vitro* studies to evaluate its toxic effects. Our results conclude that *Rheum ribes* can have some negative effects on human hepatocytes by causing cell death and DNA damage.

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**References**

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