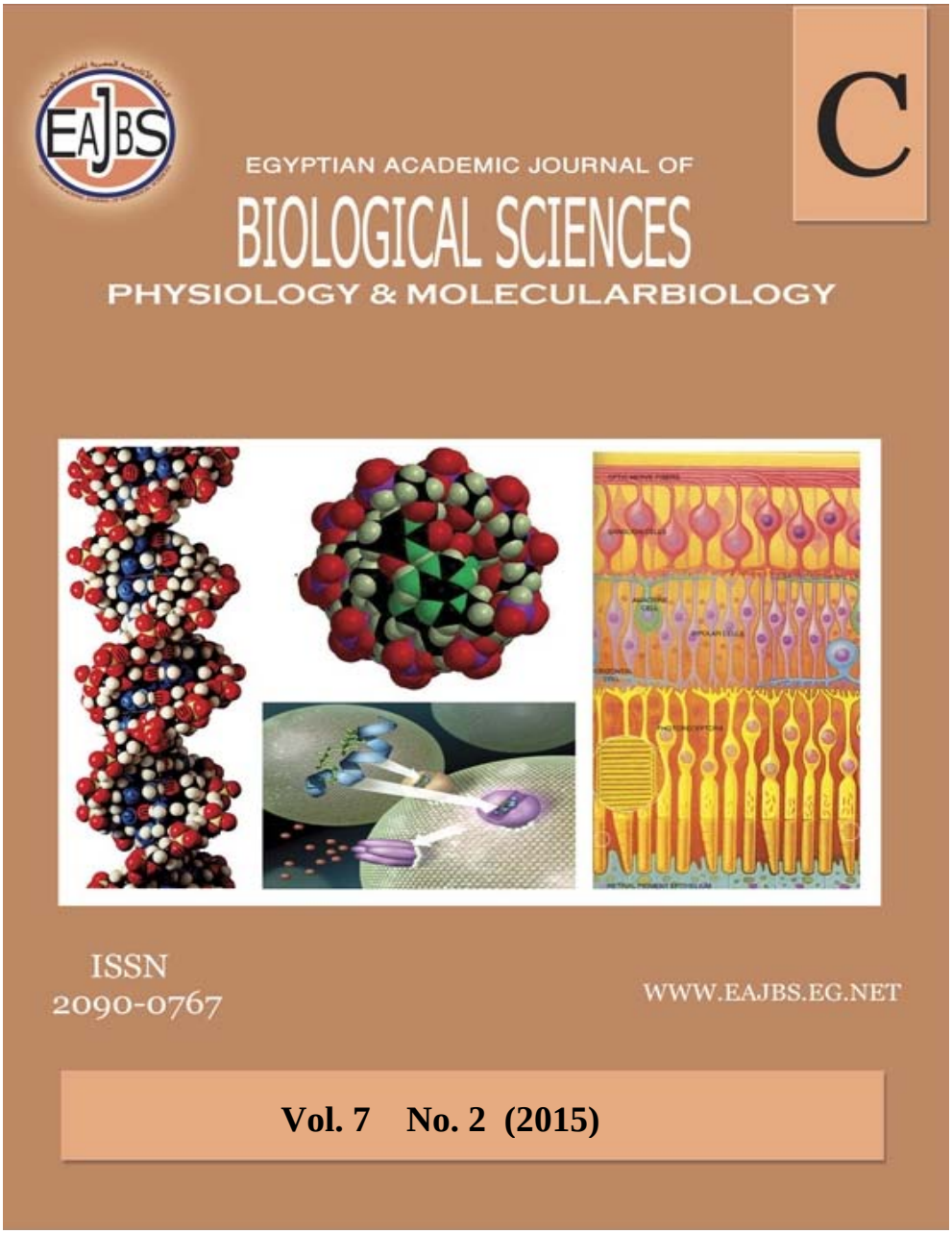

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Anti-Cancer Activity of the *Rubus idaeus* Extracts Against HepG2 and $L_{20}B$ Cell Lines Using Tissue Culture Technique

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ABSTRACT

The extracts were prepared from ripe blackberries. The extracts were tested against the human hepatocellular carcinoma cell line, HepG2, and the mouse cell line $L_{20}B$ using a neutral red assay. The extracts showed a clear inhibition rate, in a dose dependent manner in both cell lines. In addition, the inhibition rate was significantly higher ($P < 0.05$) for alcoholic extracts in both cell lines compared to aqueous extracts. The results clearly show that *Rubus idaeus* fruit extracts have potent cytotoxic and cytostatic effects on both cell lines. On the basis of these results, we can conclude that *Rubus idaeus* has a strong cytotoxic effect on both human and mouse cancerous cells.

INTRODUCTION

The fruits of *Rubus idaeus* contain a wide range of bioactive phytochemicals, including vitamin C and total phenolics [Mullen *et al*., 2002]. They also contain flavonoids, anthocyanins, and ellagitannins; and it is the flavonoids and anthocyanin pigments that give raspberries their characteristic colour [Chanjirakul *et al*., 2006]. Many of the health benefits derived from the consumption of the blackberry, *Rubus idaeus*, are due to their content of polyphenols, which are responsible for many of the biological activities of these berries, including antioxidant, anti-inflammatory, and anticancer properties, etc. [Seeram, 2008].

The anticancer properties of *Rubus idaeus* have been attributed to their content of vitamin C, anthocyanins, and ellagitannins. Vitamin C contributes about 20% of the total anticancer capacity, anthocyanins about 25%, with the largest contributor to its antioxidant capacity is made by the ellagitannins at more than 50% [Shiow, 2009]. Ellagitannins are uncommon phytochemical found in only a limited number of berry species, including in cloudberries, raspberries, and, to a limited extent, in strawberries [Beekwilder *et al.,* 2005]. Berry bio-actives, including those of the blackberry, have many roles in cancer prevention according to [Stoner *et al.,* 2008].

Laboratory studies have shown that the berry's bio-active compounds protect against oxidative DNA damage by direct scavenging of reactive oxygen species (ROS), often considered as a first line of defence against the multistage process of carcinogenesis [Nijveldt *et al*., 2001]. Berry bio-active compounds are also effective in inhibiting the formation of carcinogen-induced DNA adducts, enhancing DNA repair and inhibiting carcinogen-induced tumour-genesis in animal models [8]. In addition, berry bio-active compounds modulate the signalling pathways involved in cellular proliferation, apoptosis, inflammation, angiogenesis, and cell cycle arrest [Van Acker SA *et al.,* 1995].

The aim of this study was to evaluate for the first time in Iraq the cytotoxic and anti-proliferative effects of *Rubus idaeus* on cancer cell lines, including the human hepatocellular carcinoma cell line, HepG2, and the mouse cell line $L_{20}B$.

MATERIALS AND METHODS

Sample Preparation: Ripe blackberries from *Rubus idaeus* were collected from different farmland areas in Iraq in November 2013. After collection, the *Rubus idaeus* fruits were identified and authenticated by a plant taxonomist. The *Rubus idaeus* fruits were washed, cleaned, weighed, and preserved at-20 $^{\circ}$C for later use.

Aqueous and alcoholic extracts were prepared from blackberry juice according to [Van Acker SA *et al.,* 1995]. After preparation of the extracts, the solvent (water or alcohol) was evaporated and dry extracts were prepared, weighed, and dissolved in Dimethylsulphoxide (DMSO) in order to prepare different concentrations for the cytotoxic assay [Beekwilder *et al.,* 2005]. A *Rubus idaeus* extract stock solution was made by mixing 400 µl of extract with 10 µl of DMSO, with the volume being completed up to 1 ml using a serum free medium to obtain the concentration of 400 mg extract/1 ml medium [Shiow., 2009].

Nine crude extracts were made by serial dilution, with concentrations of 3.9, 7.81, 15.62, 31.25, 62.5, 125, and 250 mg/ml, respectively. These were added in triplicate to the microtiter plate containing 1×10^5 cells/well and 200 µl of the medium.

Culture of Cell Lines: The cytotoxicity assay for HepG2 and $L_{20}B$ was performed at the Animal Cell Culture Laboratory, Biotechnology Research Centre at Al-Nahrain University. Cells were cultured in DMEM medium supplemented with 10% foetal bovine serum, L-glutamine, Non-essential amino acids, HEPES, sodium bicarbonate, streptomycin, and penicillin. Cells were grown as a monolayer in a humidified incubator at 37 °C with 5% CO_2. The experiments were performed when cells were healthy, active, and in the logarithmic phase of growth [Shiow, 2009].

Cytotoxicity Assay: To detect the extent to which the growth of the HepG2 and $L_{20}B$ cell lines was inhibited, cells were incubated with different concentrations of each extract. The nine concentrations were used in triplicate to investigate their cytotoxic and anti-proliferative effects. A complete medium was used as negative control.

Neutral Red Assay: After incubating the cells with the extracts for 48 hours, the wells were washed with PBS and a freshly prepared neutral red solution (100µl/well) was added and incubated for 2 hrs. Finally, wells were washed again with PBS to remove excessive dye. An elution buffer (100 µl/well) was added and the absorbance was measured using an ELISA reader at a wavelength of 492 nm.

The percentage of inhibition rate (IR) was calculated according to the formula:

(%IR) = absorption at 490 nm for the control minus absorption at 490 nm for extracts divided by absorption at 490 nm for the control, with this answer being multiplied by 100 [Freshney, 2004].

RESULTS AND DISCUSSION

Cell culture can be a very sensitive and reproducible method for the preliminary screening of the inhibition rate of active ingredients on cancer cell lines [Huang *et al*., 2002], because the active ingredients can be tested on animal cells in a controlled way. Cytotoxicity assays in general are able to detect many mycotoxins that potentially inhibit the biochemical activity of numerous animal or human cell lines [Cetin *et a*l., 2005]. Several methods have been developed to assess the growth inhibition of cancer cell lines, with one example being the neutral red assay used here [Gutleb *et al*., 2002].

Statistical Analysis: The values of the investigated parameters were given as the mean ± standard error, and the results obtained were statistically analysed using Duncan's multiple range test in SAS software (version 17; SAS Inc., Chicago, IL, USA) [SAS, 2004].

The cytotoxic effect of different concentrations of the *Rubus idaeus* extracts after treating HepG2 and $L_{20}B$ cell lines for 48 hours is shown in Fig. 1.

The statistical analyses showed a significant cytotoxic effect ($P < 0.05$) from alcoholic and aqueous extracts, and it is clear from the figure that as the extract concentration increased there was marked increase in the inhibition rate against both cell lines, with this trend being almost the same for both the alcoholic and aqueous extracts of *Rubus idaeus.*

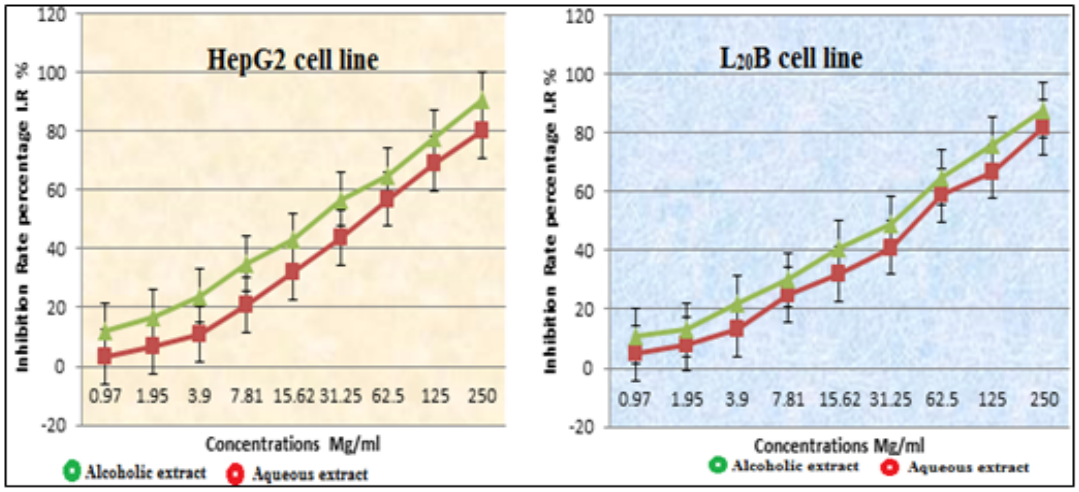

Fig. 1: The cytotoxic effect expressed as the inhibition rate percentage (%IR) for different concentrations of *Rubus idaeus* extracts after 48 hours exposure on HepG2 and $L_{20}B$ cell lines.

Figure 1 also shows that for the higher concentrations (31.25, 62.5, 125, and 250 mg/ml of extract) the toxic effect was potent, with both HepG2 and $L_{20}B$ cell lines showing inhibitions increasing from over 40% to over 90%. Against the HepG2 cell line, the alcoholic extracts gave excellent inhibition rates of 12, 17, 24, 35, 43, 57, 65, 78, and 91%, respectively for the nine tested concentrations. The performance of the aqueous extract against the HepG2 cell line was slightly inferior; however, at 3, 7, 11, 21, 32, 44, 57, 69, and 80%, respectively. Against the $L_{20}B$ cell line, the inhibition rate of the alcoholic extract was 11, 13, 22, 30, 41, 49, 65, 76, and 88%, respectively, for the nine tested

concentrations, although once again the aqueous extracts were slightly less effective with inhibition rates of 5, 8, 13, 25, 32, 41, 59, 67, and 82%, respectively. DMSO tested in the concentration used to dissolve samples and showed no signs of cell death because it is used in a concentration less than 1% DMSO at this concentration had no effect against the cells. It is evident, therefore, that both extracts had strong cytotoxic and inhibitory effects on the two cell lines used for the present study. As *Rubus idaeus* contains bioactive compounds, addition of these compounds to cells may result in cytotoxic, cytostatic, and anti-proliferative effects, causing damage to the cell membrane, and/or mutations in DNA or RNA which result in the death of cancerous cells.

The present data are in accordance with many other studies worldwide, in which similar types of extracts have been tested on cancer cell lines. The cytotoxic activity of *Rubus idaeus* extracts is mostly due to the presence of phenols, aldehydes, and alcohols in their composition [Shiow, 2009]. These compounds seem to have no specific cellular target and *in vitro* physicochemical assays have characterized most of them as antioxidants [Gutleb *et al*., 2002]. Since they are considered to be typical lipophiles, they are able to pass through the cytoplasmic membrane and can easily enter into cells and, in higher amounts, can also cause damage to the membrane. A study has shown; however, that in eukaryotic cells, alcoholic and aqueous extracts can act as pro-oxidants, affecting inner cell membranes and organelles such as mitochondria [Shiow, 2009]. Depending on their type and concentration, they exhibit cytotoxic effects on living cells but are usually non-genotoxic. In some cases; however, these compounds bring changes in the intracellular redox potential and mitochondrial dysfunction, and in this way they also have the capacity to have genotoxic effects in living systems [Gutleb *et al*., 2002].

One of the advantages of utilizing *Rubus idaeus* fruit juice is the fact that it is usually devoid of long-term genotoxic risks [Mullen *et al*., 2002]. Moreover, some compounds in the juice show a very clear anti-mutagenic capacity which could well be linked to anti-carcinogenic activity. *Rubus idaeus* active compounds have many biological activities with some of the main properties including the ability to induce apoptosis, antibacterial, hepatoprotective, vaso-relaxant activities, cyclooxygenase inhibitory effects, and an antitumor action, etc. [Nijveldt *et al*., 2001].

A study has demonstrated that *Rubus idaeus* extracts (polyphenols) are very efficient in reducing the volume of local tumours or tumour cell proliferation by inducing apoptosis. These findings suggest that, at least in part, the encountered beneficial effects of *Rubus idaeus* extracts are due to pro-oxidant effects at the cellular level [Mullen *et al*., 2002]. The present study showing that the local fruit of the *Rubus idaeus* plant exhibit cytotoxic effects on two cancer cell lines draws attention to *Rubus idaeus* as a potential anticancer drug but further investigation is required in order to elicit the exact mechanism by which these extracts act in comparison to traditional anticancer drugs.

In humans, any benefits of *Rubus idaeus* consumption relative to anticancer properties in patients need to be demonstrated. The benefits derived from raspberry consumption have been attributed to their content of polyphenols, flavonoids, anthocyanins, ellagitannins, and vitamin C. These are the phytochemicals that are responsible for many of the biological activities of raspberries, including antioxidant, anti-inflammatory, and anticancer properties [Nijveldt *et al*., 2001]. Blackberries are strong antioxidants and have a high free

radical scavenging capacity, due primarily to their ellagitannins. Among fruits and berries, raspberries contain some of the highest levels of ellagitannins, which are abundant in the pulp and seeds. So, on the basis of their huge benefits, and the fact that they contain a high number of anti-oxidant compounds, *Rubus idaeus* could be tested on cancer patients. Although the antioxidant activity of the blackberry was directly related to the total amount of phenolic and flavonoids, no relationship was established between the anti-proliferative activity and the total amount of phenolic compounds in the berries [Shiow, 2009].

CONCLUSION

There is a need for more investigational studies to elucidate the molecular mechanism of the bioactive compounds in extracts of *Rhubus idaeus* in order for it to be used as a therapeutic agent for cancer treatment.

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