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In vitro evaluation of anticancer, antiviral, and antioxidant properties of an aqueous methanolic extract of *Rhus typhina* L. leaves

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Abstract

In recent decades, natural products of plant origin have predominated among the sources of new therapeutic agents. Plants, which are intensively used in folk medicine, are the focus of particular interest, and being subjected to in-depth analyses for the assessment of their pharmacological properties. The objective of the present work was to evaluate the *in vitro* anticancer, antiviral, and antioxidant properties of a crude aqueous methanolic extract from the leaves of *Rhus typhina* L., which is a plant species with broad applications in traditional phytotherapy. The anticancer capacity of the extract was assessed on two human breast cancer cell lines (MCF7 and MDA-MB-231) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay, and its cell growth inhibitory effect on cancer cells was compared with its action on a non-cancerous breast epithelial cell line (MCF10A). Moreover, the *in vitro* antiviral activity of the plant extract was studied against viral strains of three taxonomic groups: herpes simplex virus type 1 (HSV-1), coxsackievirus B1 (CVB1), and influenza A virus (IAV/H3N2) using a cytopathic effect inhibition test, extract cytotoxicity evaluation through the neutral red uptake assay, a virucidal assay, a viral attachment assay, and cell pre-treatment with the extract. In turn, a radical scavenging assay with 2,2-diphenyl-1-picrylhydrazyl (DPPH) was applied to explore the antioxidant potential of *R. typhina* leaf extract. Results indicated that the plant extract possessed antiproliferative activity against the tested cell lines which was stronger toward cancer cells. A modest inhibitory effect on IAV/H3N2 replication was detected; as well as on an effect on herpes virions and on the adsorption of the HSV-1 and IAV/H3N2 strains; and a protective effect on uninfected cells before HSV-1 contact. The leaf extract of *R. typhina* also exhibited a strong free radical scavenging activity. The results of the present work demonstrated the pharmacological potentials of the plant extract, which warrants further and more detailed study in the future.

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Introduction

Plants, as a valuable source of bio-active compounds, are used worldwide, not only as a traditional remedy for many health conditions, but also in modern pharmacy for the production of medicinal products. It is estimated that about 25% of all medicines that are used in clinical practice worldwide are directly or indirectly derived from higher plants (Gurib-Fakim, 2006). *Rhus typhina* L. (Anacardiaceae), which is commonly known as staghorn sumac, is a large deciduous shrub. Although North America is the natural area of distribution of this species, it is also widely cultivated in temperate regions. The plant has been reported to possess antiseptic, tonic, diuretic, stomachic, antidiabetic, antioxidant, and antimicrobial properties (McCune and Johns, 2002; Kossah *et al.*, 2009). Various potential applications of the plant in the food, nutraceutical, and cosmetic industries have been

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suggested based on its active compounds. The chemical composition of *R. typhina*, which is affected by genetic and environmental factors, plant parts, and extraction methods, includes polyphenols (gallic, caffeic, and ellagic acids; anthocyanins), organic acids (malic, citric, tartaric, and fumaric acids), essential amino acids, unsaturated fatty acids, minerals, vitamins, dietary fibres, and other components (Wang and Zhu, 2017).

The data available on the anticancer capacity of *R. typhina* extracts stems from several *in vitro* studies, the first of which analysed the effect of a crude leaf aqueous extract on mouse leukaemia cells (Guinot *et al*., 2010); whereas another study compared the cell growth inhibitory effect of fruit extracts of *R. typhina* and raspberry, alone and in combination, on rat colon and human breast cancer cells (Wang *et al*., 2015a). Another study from the same authors focused on the assessment of the cytotoxic activity of *R. typhina* fruit extract individually and in combination with the chemotherapeutic agent 5-fluorouracil (Wang *et al*., 2015b). Recently, Wang *et al*. (2019) provided information about the activity of *R. typhina* fruit extract against two human colon cancer cell lines.

To the best of our knowledge, no data are currently available regarding the antiviral activity of *R. typhina* extracts. Limited information has been reported in respect to other representatives of the genus *Rhus*, including a study that detected an inhibitory effect of several biflavonoids isolated from the seed kernels of *R. succedanea* against influenza A and B viruses, HSV-1, HSV-2, and measles (Lin *et al*., 1999). Another publication reported a strong *in vitro* antiviral activity of an aqueous extract of the root/stem bark of *R. aromatica* against HSV-1 and HSV-2 (Reichling *et al*., 2009).

Regarding the antioxidant behaviour of the components of *R. typhina*, several studies have evaluated the potential of different types of extracts. Antioxidant activity of the fruit extract of the plant was detected by Kossah *et al*. (2011). A recent study reported that the stem of *R. typhina* possessed high antioxidant capacities due to its high phenolic content (Liu *et al*., 2019). Wu *et al*. (2013) revealed that *R. typhina* had a stronger *in vitro* antioxidant activity than blueberry and cranberry fruits, which are known to have strong antioxidant properties due to their phytochemicals, including gallic acid, ellagic acid, caffeic acid, quercetin, uncommon anthocyanins, and other components. Another study found that the polyphenols isolated from the plant crude extract exhibited *in vitro* antioxidant activity on the Caco-2 colon cancer cell line (Peng *et al*., 2016).

Considering the diverse biological activities of *R. typhina* and the increasing interest in its potential application as a functional food, more intensive studies on its pharmacological properties are required. The present work was undertaken to generate data on the anticancer capacity of *R. typhina* leaf aqueous methanolic extract against two breast cancer cell lines, and to compare the effect of the extract on the growth of a non-cancerous cell line. Finally, the antiviral potential of *R. typhina* extract against herpes simplex virus type 1, coxsackievirus B1, and influenza virus A, and its antioxidant free radical scavenging activity were also assessed.

Materials and methods

Plant materials and extraction procedure

Leaves of *R. typhina* were collected from individuals cultivated in park areas of Sofia, Bulgaria. The studied materials consisted of 50 g of bulk sample from the leaves of five individual trees (10 g each). The species was identified according to Palamarev (1979).

The air-dried and ground plant material was extracted with methanol *via* classical maceration for 24 h, three times. After evaporation of the solvent, the resulting crude extract was subjected to subsequent analyses.

Cell lines and cell culturing

The human breast adenocarcinoma cell lines MCF7 and MDA-MB-231, and the non-cancerous breast epithelial cell line MCF10A were purchased from the American Type Culture Collection (ATCC; Manassas, Virginia, USA). MCF7 and MDA-MB-231 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% sodium pyruvate, and 1% MEM Non-Essential Amino Acids (NEAA); while MCF10A cells were cultured in DMEM medium containing 5% FBS, 1% sodium pyruvate, 1% NEAA, 20 ng/mL of human epidermal growth factor (hEGF), 10 μg/mL of insulin, and 0.05 mM hydrocortisone.

Madin-Darby bovine kidney (MDBK) cells and human epithelial type 2 (HEp-2) cells (from human laryngeal carcinoma) were obtained from the National Bank for Industrial Microorganisms and Cell Cultures, Sofia. Madin-Darby canine kidney

(MDCK) cells were purchased from the ATCC. The three cell lines were grown in DMEM containing 10% FBS and supplemented with 10 mM HEPES buffer and antibiotics (penicillin, 100 IU/mL; streptomycin, 100 μg/mL).

All cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Viruses

Herpes simplex virus type 1, Victoria strain (HSV-1) was obtained from Prof. S. Dundarov, National Centre of Infectious and Parasitic Diseases, Sofia. The virus was replicated in monolayer MDBK cells in a maintenance solution of DMEM containing 0.5% FBS. The infectious virus titre was $10^{6.75}$ $CCID_{50}$/mL.

Coxsackievirus B1 (Connecticut 5 strain, CVB1) was obtained from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), and propagated in HEp-2 cells (in a maintenance solution of DMEM with 10 mmol/L HEPES, 0.5% FBS, 100 IU/mL of penicillin, and 100 mg/mL of streptomycin). The infectious virus titre was $10^{6.5}$ $CCID_{50}$/mL.

Influenza virus A/Panama/2007/99/H3N2 (IAV/H3N2) was obtained from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), from the allantoic fluids of virus-inoculated 10-day-embryonated eggs, incubated at 37°C. The infectious virus titre was $10^{6.0}$ $CCID_{50}$/mL.

MTT cell proliferation assay

Cell proliferation was assessed using the MTT colorimetric assay (Mosmann, 1983). The MCF7, MDA-MB-231, and MCF10A cell lines were seeded into 96-well microplates at a density of 5,000 cells/well in a final volume of 200 μL, and attached to the bottom of the well overnight. On the following day, the medium was replaced with fresh medium containing increasing concentrations of the studied extract in a range of 10 - 180 μg/mL, followed by incubation for 72 h. Cell samples with medium were used as negative controls. In the last 4 h of the incubation, 20 μL of MTT solution (5 mg/mL) was added to each well after which the medium was removed. The optical density (OD) was measured at 570 nm using a microplate reader (Thermo Scientific Multiskan Spectrum) after the formazan complex was dissolved in 100 μL of 10% SDS, 0.01 M HCI. The percentage of cell proliferation after treatment with the extract was determined using Eq. 1:

$$\text{Cell proliferation (\%)} = [OD_{\text{test sample}} / OD_{\text{control}}] \times 100 \quad \text{(Eq. 1)}$$

Data were presented as mean ± standard error of the mean (SEM) of three separate experiments, each of which was performed in at least three parallel repeats. The half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). The statistical significance of the differences between the control and the treatment groups was evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Significance was set at $p < 0.05$.

To evaluate the degree of selectivity of the antiproliferative action of the plant extract, the selective index (SI) was calculated using Eq. 2:

$$SI = IC_{50\ \text{non-cancerous cell line}} / IC_{50\ \text{cancer cell line}} \quad \text{(Eq. 2)}$$

Microscopic assessment of cell morphology

The cell morphological alterations that occurred after treatment with *R. typhina* extract were observed using an inverted light microscope (Carl Zeiss) in parallel with the MTT cell proliferation assay.

Neutral red cytotoxicity assay

The *in vitro* cytotoxic effects of the crude extract of *R. typhina* were examined using MDBK, HEp-2, and MDCK cells. A confluent cell monolayer in a 96-well plate was treated with culture medium containing no (untreated control) or increased concentrations of the compounds. The cells were incubated at 37°C and 5% CO_2 for 48 h. After microscopic evaluation, the cells were washed, and 0.1 mL of maintenance medium supplemented with 0.005% neutral red dye was added to each well, followed by incubation at 37°C for 3 h. Subsequently, the neutral red dye was removed, the cells were washed once with phosphate-buffered saline (PBS), and 0.15 mL of desorb solution (1% glacial acetic acid and 49% ethanol in distilled water) was added per well. The OD of each sample was measured at 540 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Biotek Organon, West Chester, PA, USA). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced the cell viability by 50% when compared with the untreated control. The maximum tolerable concentration (MTC) of a substance is the highest concentration of

the compound that does not cause the death of the treated cells.

Antiviral activity assay

The antiviral screening was based on the viral yield reduction technique. The cytopathic effect (CPE) inhibition test used a confluent cell monolayer in 96-well plates infected with 100 $CCID_{50}$ in 0.1 mL. After 1 h of virus adsorption, the extract was added at various concentrations, and the cells were incubated for 48 h at 37°C. The cells were then stained and their viability was estimated based on the neutral red uptake assay of an ELISA reader at OD_{540nm}. The percentage of CPE inhibition for each concentration of the test sample was calculated using Eq. 3:

$$\% \text{ CPE} = [OD_{\text{test sample}} - OD_{\text{virus control}}] / [OD_{\text{toxicity control}} - OD_{\text{virus control}}] \times 100 \quad \text{(Eq. 3)}$$

The 50% inhibitory concentration (IC_{50}) was defined as the concentration that inhibited 50% of the viral replication when compared with the virus control. The SI was calculated based on the CC_{50}/IC_{50} ratio.

Virucidal assay

Samples (1 mL) containing CBV1 or HSV-1 (10^4 $CCID_{50}$) and the tested compound at its MTC (with different values for the individual cell lines, respectively: for MDBK, 80 µg/mL; for HEp-2, 100 µg/mL) were contacted in a 1:1 ratio, and subsequently stored at room temperature for different time intervals (15, 30, 60, 90, and 120 min). Subsequently, the infectious viral titre of all of the contact samples was determined *via* end-point dilution titration. The results were compared to those obtained from the virus control, which consisted in an equal volume of virus suspension and maintenance medium, incubated for the same time intervals. Finally, Δlog was calculated.

Virus attachment assay

Monolayers of MDBK, HEp-2, or MDCK cells in 24-well cell culture plates (pre-chilled at 4°C) were inoculated with 10^4 $CCID_{50}$ of HSV-1, CBV1, or IAV/H3N2 at 4°C, and treated in parallel with the MTC of the extract (for MDBK, 80 µg/mL; for HEp-2, 100 µg/mL; and for MDCK, 139.5 µg/mL). At different time intervals (15, 30, 45, and 60 min), the cells were washed with PBS to remove both the compound and the unattached virus, then overlaid with maintenance medium and incubated at 37°C for 24 h. After three rounds of freezing and thawing, the infectious virus titre of each sample was determined *via* the end-point dilution method.

Pre-treatment of MDBK cells

MDBK cell monolayers in 24-well cell culture plates (2×10^6 cells per well) were pre-treated for 15, 30, 60, 90, and 120 min at the MTC (80 µg/mL) of the extract in the maintenance medium (1 mL per well). Subsequently, the cell culture media containing the substance were removed, and the cells were washed twice with PBS, and inoculated with HSV-1 (1000 $CCID_{50}$ in 1 mL per well). After 60 min of absorption, the virus was removed, and the cells were covered with maintenance medium. The culture plates were incubated at 37°C for 24 h, and after three rounds of freezing and thawing, the infectious virus titres were determined using the end-point dilution method. The results were compared with those obtained for the virus control which consisted of cells infected with the virus but not pre-treated with the extract. Finally, Δlog values were determined.

Antioxidant DPPH radical scavenging activity

The stable 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) was used for the determination of the free radical scavenging activity of *R. typhina* extract (Stanojević *et al*., 2009). Different concentrations of the extract were added at an equal volume (2.5 mL) to a methanol solution of DPPH (0.3 mM, 1 mL). After incubation for 30 min at room temperature, the OD was measured at 517 nm using a spectrophotometer (Jenway 6320D), then converted to the percentage of antioxidant activity using Eq. 4:

$$\text{DPPH antiradical scavenging capacity (\%)} = [1 - (OD_{\text{sample}} - OD_{\text{blank}}) / OD_{\text{control}}] \times 100 \quad \text{(Eq. 4)}$$

Methanol (1.0 mL) plus the plant extract solution (2.5 mL) was used as a blank, whereas the DPPH solution plus methanol was used as a control. Measurements of each sample were carried out in triplicate, and the data were presented as the mean ± SEM.

Results

Anticancer activity of R. typhina extract

The antiproliferative properties of *R. typhina* extract were tested on two human breast cancer cell

lines, *i.e.*, MCF7 (oestrogen and progesterone receptor-positive and human epidermal growth factor receptor 2 (HER2)-negative) and MDA-MB-231 (oestrogen receptor-negative, progesterone receptor-negative, and HER2-negative) cells, and compared with the non-cancerous cell line MCF10A after treatment for 72 h with extract concentrations ranging from 10 to 180 µg/mL using the MTT cell proliferation assay.

Results showed that *R. typhina* extract significantly inhibited the growth of the two breast cancer cell lines ($p < 0.01$ at 10 µg/mL for MCF7 and $p < 0.0001$ at all other tested concentrations), with IC_{50} values of 56.8 µg/mL for MCF7 cells, and 169.1 µg/mL for MDA-MB-231 cells (Figure 1).

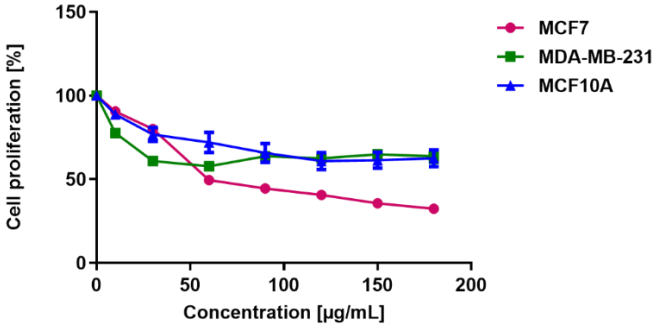

Figure 1. MTT cell proliferation assay of MCF7, MDA-MB-231, and MCF10A cells treated with increasing concentrations of *Rhus typhina* extract for 72 h. Error bars represent the standard error of the mean (SEM).

However, although the proliferation of hormone-independent MDA-MB-231 breast cancer cells was less affected by the extract when compared with the MCF7 hormone-dependent cells, it should be noted that at the lowest concentrations of 10 and 30 µg/mL, the MDA-MB-231 cell line was more sensitive to treatment than was the MCF7 cell line. The induction of cell morphology alterations, and the decrease in the density of the cell monolayer after extract treatment were observed in both cancer cell lines using light microscopy (Figure 2).

The non-cancerous MCF10A cell line exhibited a more modest reduction in growth when compared with the cancer cell lines at lower concentrations, from 10 to 90 µg/mL (at 30 µg/mL, $p < 0.01$; at 60 µg/mL, $p < 0.001$; and at 90 µg/mL, $p < 0.0001$); in contrast, at higher doses of up to 180 µg/mL, the inhibitory effect of the extract was slightly stronger than that recorded for the MDA-MB-231 cell line (at 120, 150, and 180 µg/mL, $p < 0.0001$). The IC_{50} value obtained for the non-cancerous cell line was 201.9 µg/mL. Morphological changes were also observed at the highest concentrations tested (Figure 2).

The SI was calculated by comparing the IC_{50} value of *R. typhina* extract in the MCF10A cell line against the IC_{50} values of the extract in the MCF7 and MDA-MB-231 cancer cell lines. Higher SI was obtained for the MCF7 (3.6) *vs*. the MDA-MB-231 (1.2) cell line.

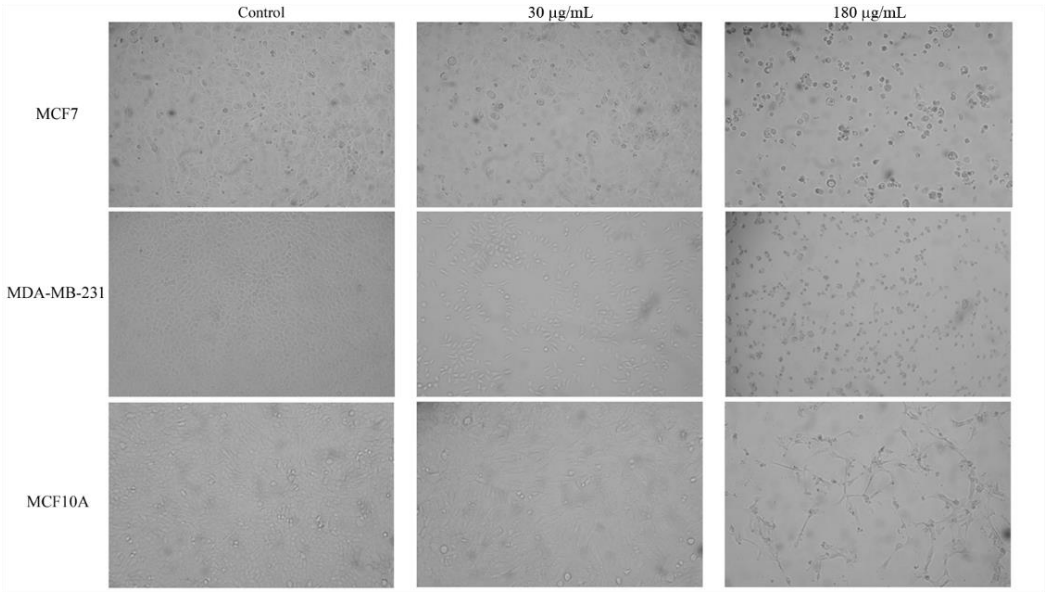

Figure 2. Changes in the morphology of MCF7, MDA-MB-231, and MCF10A cells after treatment with *Rhus typhina* extract at 30 and 180 µg/mL for 72 h as compared to control.

Antiviral activity of R. typhina extract

For the accuracy of all experiments carried out using viral strains, it was necessary to determine in advance the doses at which the extract was non-toxic to the cells to which it was administered. For this purpose, the cytotoxicity of the extract was determined against the MDBK, HEp-2, and MDCK cells used in subsequent antiviral experiments. The results of the test of the cytotoxicity of *R. typhina* extract are presented in Table 1.

The findings obtained with respect to the three cell lines revealed a similar cytotoxicity, with the lowest toxicity observed against MDCK cells and the highest observed against the MDBK cell line.

Testing of the antiviral activity of *R. typhina* extract revealed a slight inhibition of IAV/H3N2 replication, with SI of 9.7; however, no specific antiviral effect was detected against HSV-1 and CVB1 replication (Table 1).

Table 1. *In vitro* cytotoxic and antiviral activities of *Rhus typhina* extract.

Cytotoxicity, CC_{50} (µg/mL)			Antiviral activity					
			CVB1		HSV-1		IAV/H3N2	
HEp-2	MDBK	MDCK	IC_{50} (µg/mL)	SI	IC_{50} (µg/mL)	SI	IC_{50} (µg/mL)	SI
207	106	279	-	-	-	-	28.7	9.7

After establishing that the extract had no effect on HSV-1 and CVB1 after they had invaded the cells, we examined its effect on the extracellular virions of both viruses. The results presented in Table 2 indicate that, in direct contact with extracellular HSV-1 virions, an effect was observed at a time point as early as 15 min of exposure, with Δlog = 1.5. The activity increased at 60 min, with Δlog = 1.75, and the strongest effect was observed at 120 min, with Δlog = 2.0, thus indicating that the effect of the extract on herpes virions was time-dependent.

No effect was observed upon the interaction of the extract with the extracellular virions of CVB1 at 15 and 30 min. A low activity was observed after 60 min (Δlog = 0.5; Table 2), and this value was maintained over the remaining time intervals studied.

Table 2. Virucidal activity of *Rhus typhina* extract.

Virus	Δlog				
	15 min	30 min	60 min	90 min	120 min
HSV-1	1.5	1.5	1.75	1.75	2.0
CVB1	0	0	0.5	0.5	0.5

The effect of *R. typhina* extract on the attachment of virions to sensitive cells is presented in Table 3. An inhibitory effect was observed against the adsorption of HSV-1; moreover, at the first test interval (15 min), we observed that Δlog = 1.5, and the number of HSV-1 virions adsorbed on the cell decreased as the duration of the exposure increased, with Δlog values at 45 and 60 min of 1.75 and 2.75, respectively. The activity of *R. typhina* extract on the adsorption of IAV/H3N2 at 15 min was comparable to that observed for HSV-1 at the same interval of action, with Δlog = 1.33; however, this action decreased with the increase in the duration of the exposure, with Δlog = 1 at 60 min. No effect on the adsorption of CVB1 was observed for any of the intervals of exposure to the extract.

Table 3. Viral adsorption of *Rhus typhina* extract.

Virus	Δlog			
	15 min	30 min	45 min	60 min
HSV-1	1.5	1.5	1.75	2.75
CVB1	0	0	0	0
IAV (H3N2)	1.33	1.33	1.33	1.0

Having found that the tested extract affected the extracellular virions of HSV-1 and significantly inhibited the stage of their adsorption onto sensitive cells, we then investigated its protective effect in uninfected cells before they had contact with the virus. Cells treated with the extract exhibited a significant decrease in the infectious viral titres. At the first studied time interval (*i.e.*, 15 min), this decrease was associated with Δlog = 2.0, which

remained constant up to 90 min. At 120 min, the effect was slightly enhanced, to Δlog = 2.25.

Antioxidant activity of the R. typhina extract

The methanolic solution of *R. typhina* extract was investigated for free radical scavenging activity using the DPPH assay. High antioxidant activity of the extract was observed in the concentration range of 10 - 200 μg/mL, from 85.3 to 94.7%, respectively, with an IC_{50} < 10 μg/mL (Figure 3).

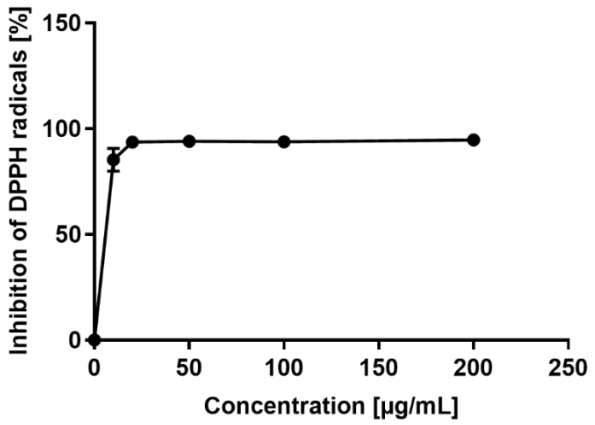

Figure 3. Free radical scavenging activity of *Rhus typhina* extract as assessed using the DPPH assay. Error bars represent the standard error of the mean (SEM).

Discussion

Currently, about 35,000 plant species are used for medicinal purposes worldwide (Yirga *et al*., 2011). The need for the study of the therapeutic properties and mechanisms of action of medicinal plants is crucial to avoid their misuse.

Breast cancer is one of the most frequently diagnosed and lethal cancer types, with about 2.1 million new cases and 627,000 deaths reported among women in 2018, according to the World Health Organization (Bray *et al*., 2018). It represents a heterogeneous disease with high rates of diversity and variability in the response to different therapies. Despite the considerable effort toward and advances in cancer therapy during recent decades, more effective and less detrimental treatment opportunities are required. Regarding the anticancer properties of the crude aqueous methanolic extract of *R. typhina* leaves, the results presented herein revealed stronger antiproliferative properties (lower value of IC_{50} concentration) and higher selectivity (greater SI) of the extract toward the hormone-sensitive MCF7 breast cancer cell line when compared with the triple-negative MDA-MB-231 breast cancer cell line. The anticancer potential of *R. typhina* is scarcely investigated. Guinot *et al*. (2010) reported that an aqueous extract of *R. typhina* leaves inhibited the proliferation of L1210 mouse leukaemia cells with an IC_{50} value of 250 μg/mL. A study from Wang *et al*. (2015a) provided data on the enhanced synergistic effect of extracts from *R. typhina* and raspberry fruit against the proliferation of the human MCF7 breast cancer cell line and the D/V-Src rat colon cancer cells, as well as its weaker cytotoxicity against the human MCF10A non-cancerous breast epithelial cell line and normal rat colon 4D/WT cells when compared with the individual application of the extracts. In another publication (Wang *et al*., 2015b), the same authors revealed that *R. typhina* fruit extract in conjunction with the oncotherapeutic drug 5-fluorouracil increased the cytotoxicity to MCF7 cells, but had a protective effect toward the MCF10A cell line; moreover, when used alone, the plant extract exhibited a selective dose-dependent inhibitory effect on MCF7 cells. Another very recent study (Wang *et al*., 2019) of the antiproliferative activity of the fruit extract and fractions of *R. typhina* on two colon cancer cell lines (HEK293 and HT-29) revealed that the ethyl acetate fraction of the extract possessed a potent growth inhibitory effect on HT-29 cells.

To apply a substance in clinical practice against a particular viral agent, the drug must be able to specifically affect one or more stages of viral replication. The first stage that can be modulated is the time before the entry of the pathogen into the cell, *i.e*., the extracellular virions. The next step would consist affecting the cell in a way that it becomes unsusceptible to infection with the virus. Another target step is the inhibition of the attachment of the virus to the cell, and drugs that block various essential structures within the cell that are necessary for its reproduction being most effective.

Our study revealed that to a certain extent, *R. typhina* extract specifically affected the stage of the intracellular replicative cycle of IAV/H3N2. The investigated extract represented a complex of many biologically active substances with various properties. Some of these substances are likely to bind to specific viral proteins of the HSV-1 supercapsid, thereby inactivating it because of the virucidal activity of the extract.

Some of the components of the extract probably bound to the cell receptors that recognise the viral particle, or to other parts of the cell membrane that are responsible for the attachment of the virus to the cell, which explained its effect in the condition of pre-treatment of cells and its effect on the adsorption of HSV-1 and IAV/H3N2. The inverse time dependence of IAV/H3N2 (*i.e*., the decrease in the effect with the increase of time) observed in the present work indicated that some of the bonds that were formed were most likely temporary, and eventually broke apart. The use of such preparations before viral infection could protect the cells from subsequent infection.

Regarding the available data on the antioxidant activity of *R. typhina* extracts, Kossah *et al*. (2011) established that the IC_{50} values for the fruit extract of *R. typhina* grown in China after the implementation of the DPPH and reducing power assays were 16 and 41 μg/mL, respectively. The high free radical scavenging activity detected in the present work was characterised by a lower IC_{50} value when compared with that reported by Kossah *et al*. (2011).

Conclusion

The present work demonstrated that an aqueous methanolic extract of the leaves of *R. typhina* possessed antiproliferative properties against the MCF7, MDA-MB-231, and MCF10A cell lines; however, the cell growth inhibitory effect was stronger toward the cancer cell lines when compared with the non-cancerous cells. The plant extract weakly inhibited IAV/H3N2 replication, exhibited virucidal activity against HSV-1, affected the adsorption of HSV-1 and IAV/H3N2, and had a protective effect on uninfected cells before HSV-1 contact. Moreover, the extract had a considerable free radical scavenging activity. The results obtained in this study provided information on the pharmacological potential of *R. typhina* extract; nevertheless, additional and more detailed studies of the mechanisms underlying the action of this extract are necessary.

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