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Studies of cytotoxic activity of substances obtained from leaves and roots of Sweet Flag (Acorus calamus L.) on human hepatocellular carcinoma (HepG2) in vitro

Lyudmyla V. Derymedvid[1], Lyudmyla A. Korang[1], Luba O. Shakina[2], Kateryna O. Kalko[3], Maksym Yaremenko[4], Moeen F. Dababneh[5], Oleg Gerush[6], Bogdan Nataliia*[6], Yevhen V. Bondariev[1]

1Department of Pharmacology of the National university of pharmacy of the Ministry of Health of Ukraine, 53 Pushkinska Str., Kharkiv 61002, Ukraine
2Department of human anatomy and physiology of the National university of pharmacy of the Ministry of Health of Ukraine, 53 Pushkinska Str., Kharkiv 61002, Ukraine
3Department of Clinical Pharmacology of the Institute for Advanced Training of Pharmacy Specialists National university of pharmacy of the Ministry of Health of Ukraine, Maidan Zakhysnykiv of Ukrayiny, Kharkiv, 61000, Ukraine
4Department of Botany of the National university of pharmacy of the Ministry of Health of Ukraine, 53 Pushkinska Str., Kharkiv 61002, Ukraine
5Middle East University, Queen Alia Airport Street, Amman, Jordan
6 Department of Pharmacy of the Bukovinian State Medical University Chernivtsi, 58000, Ukraine

*nataliabogdan1602@gmail.com

Abstract

"In vitro" studies play a very significant role in studies of modern toxicology. This type of researches reduces the time and cost of preliminary studies, it can be easily reproduced, it allows to introduce accurately the amount of studied substances entering the cell, and most importantly – it may become an alternative to using animals in further researches. The Replacement principle means that the research is carried out on the cell cultures, tissues, etc., using computer models without conducting experiments on animals.

Studying basic cytotoxicity plays a very important role in most researches conducted worldwide. The toxicity could be identified by damaging the cell membranes and/or causing dysfunctions of mitochondria.

Keywords: toxicology, cytotoxic, Molecular Biology, MTT assay

Introduction

Back in 1959, British scientists Russell W.M.S and Burch R.L. proposed the concept of "three Rs". "The Three Rs" means: Replacement, Reduction and Refinement [17,18,19].

A well-known model - the Primary Culture of Human Hepatocytes could be used to study the cytotoxic effect on the cells of various substances (including medicinal substances) [5]. since differentiated liver cells are able to proliferate in vitro and react to the pathophysiological factors. So, according to the Maurel's research9, hepatocytes can be functioning under favorable cultivation conditions In Vitro similarly to the basic biochemical functions of the liver parenchyma (in vivo), as well as metabolizing drugs. However, non-availability of human hepatocytes and/or its shortages, their very short life span, high cell variability during cultivation and a rapid decrease in metabolic activity extremely limit their use [8].

As an alternative model to Human Hepatocytes, several lines of Human Hepatitis Cells have been introduced in recent years, such as HepG2, Hep3B, HepaRG4. Comparing with liver cells in adults, cells of these lines have shown less differentiated phenotype and lower metabolic activity7. That's the disadvantage of the method. However, these lines have a number of advantages: a fairly stable phenotype, the preservation of many functional characteristics of differentiated liver cells, an unlimited cultivation period and good repeatability of the results when evaluating the effect of drugs in vitro [7,6].

The aim of this study was to identify cytotoxicity and determine the severity of the cytotoxic activities of Sweet Flag (Acorus calamus L.) extracts on Human Hepatocellular Carcinoma cells (HepG2) in vitro.

The extracts from rhizomes (REA) and leaves (LEA) of Acorus calamus were obtained at the Department of Botany of the National University of Pharmacy (Kharkov, Ukraine) under supervision of Prof. Gontova T. N.

To study in vitro cytotoxicity of alcohol-water extracts from the leaves and roots of Acorus Calamus the MTT method was applied10This test is the most commonly used for in vitro assessment of chemical cytotoxicity and it is also included in most protocols of Molecular Biology and Medicine[3,12,2]. And also it has been reported to be the most sensitive test in detecting cytotoxic events 13 The principle of the MTT assay is based on the ability of the dehydrogenase enzyme, produced by the mitochondria of the living cells13, to convert/reduce the yellow water-soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenylte-trazolium bromide (MTT) to purple formazan crystals insoluble in water. So the viable cells are able to reduce the yellow MTT to a water-insoluble purple substance which precipitates in the cellular cytosol and could be dissolved after cells lysis, whereas the dead cells or the cells damaged by toxins, cannot transform/reduce MTT. This reaction is proportionate to the number of viable cells and inversely proportional to the degree of cytotoxicity. The darker the solution - the less toxic the substance is.

Methods

Cell Cultures and Reagents

The cells of Human Hepatocellular carcinoma (HepG2) were obtained from the R. E. Kavetsky's Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (BioWest, France) supplemented with 10 % fetal bovine serum (FBS) (BioWest, France) and maintained at 37°C in a humidified atmosphere of 5 % CO_2 plus 95 % air.

The MTT solution: 0.5g of MTT and 100 mls of sterile Normal Saline was filtered, sterilized and kept at - 20 °C before use.

Solubilizing reagent: 997 ml of 2 - Propanol was mixed with 3mls of concentrated Hydrochloric Acid and kept at -20°C before use.

Trypan blue 0.4% solution: 40 mg of Trypan Blue and 10mls of Normal Saline was filtered and kept at room temperature before use.

Results

Preparation of alcohol-water extracts from the leaves and roots of Sweet Flag (Acorus Calamus)

To obtain different concentrations of LEA and REA, the initial extracts (concentration 0.6 g/mL) were titrated on an immunological plate by the rolling method. Normal saline (0.9% NaCl) was used as a solvent. The following concentrations of studied substances were investigated: 0.03; 0.015; 0.007; 0.004; 0.002; 0.001g/mL. In the research, concentrations less than 0.03g/mL were examined, taking into account the previously received data concerning significant cytotoxic effect of REA in the range of 1.65-0.05g/mL, which lead to an increase in the number of dead rat red bone marrow cells by 86.0-52.0%, respectively, even with short-term exposure (90 min).

Viability Assay

Before performing the MTT assay, cells viability exclusion test was conducted using Trypan Blue dye in the Goryaev chamber. Prior to staining, 20 µL of cell suspension was transferred to a microscope slide. An equal volume of 0.4% Trypan blue in Normal saline 0.9% (ie, 10 µL) was added, and the mixture was incubated at the room temperature for 1-2 min. A special glass coverslip was placed on the top of the slide to make a chamber so that the mixture completely fills the chamber. The chamber was observed under a microscope, and both: the viable (clear) cells and non-viable (blue) cells were counted. The total number of cells per mL was derived from the formula

$$\frac{\text{Total number of cells in the whole chamber} \times \text{dilution factor (ie, 2)} \times 1000}{0.9}$$

Where 1000 is the number of cubic microliters in 1 mL (1cm3), 0.9 is the chamber volume in microliters.

And the overall number of cells per mL was the mean derived from the three repeat counts. The cell viability (%) was calculated using the formula

$$\frac{\text{Number of viable cells} \times 100\%}{\text{Total number of cells counted}}$$

And the overall cell viability was the derived from the three repeat counts. It should be noted that the HepG2 cells viability test should be conducted immediately prior seeding 96-well plate and it should be as high as 85 – 90%. Finally harvested HepG2 cells were resuspended in DMEM to a final concentration of 1.0-1.1×105 viable cells per milliliter.

MTT Assay

The cytotoxicity of samples on HepG2 cells were determined by the MTT assay. Cells (1.0-1.1×105 per milliliter) were seeded in 95 µL of medium/well in 96-well plates (SPL, Korea) with 5 µL of LEA or REA extracts of the Sweet Flag. A quantitative assessment of the cytotoxicity of the samples was analyzed after 48, 72, 96 hrs of incubation with the studied substances14 at 37°C in a humidified atmosphere of 5% CO2 plus 95% air. At the same time, control samples of cells were incubated, in which sterile Normal Saline (0.9% NaCl) was added instead of extracts. The experiment was repeated in triplicate.

To start the coloring reaction, 20 µL of MTT solution was added to growing cultures and mixed well by micropipette and left for 4 hrs (20°C in the dark). Presence of viable cells was visualized by the development of purple color due to formation of formazan crystals. The supernatant was removed carefully and 100 µL of solubilizing reagent was added to the cells and mixed well by micropipette.

The 96-well plate was transferred to the spectrophotometer STAT FAX 303 plus and the OD (optical density) values were read at 492 nm with 630 nm. The optical density of the solutions is proportional to the content of living cells in them. Cell survival was calculated by the formula:

Cell viability (%) = Mean OD (wells with studied substance) / Mean OD (wells without studied substance) x 100%

Statistical Analysis

The data obtained during this research was analyzed using the statistical program Statistica 11.0 with the determination of arithmetic mean values (M), arithmetic mean errors (m), and significant differences between the comparison groups. Firstly, the reliability of differences in optical density in the experimental wells from the control wells was evaluated, then the proportions of surviving cells in each cell of the plate were calculated. As a result, each sample concentration was characterized by a set of values of the proportion of surviving cells.

Differences were considered statistically significant when p<0.05 13

Discussion

Obtained results of the cell viability of Human Hepatocellular Carcinoma (HepG2) in the MTT test showed that the ability of the cell culture to restore tetrazolium was reduced in the presence of aqueous extracts from the rhizomes of Acorus Calamus and was dose- and time-dependent.

The results of the cells (HepG2) viability study in the presence of aqueous extracts from the root of Acorus Calamus are presented in Figure 1-2.

Aqueous solutions of the substance obtained from the rhizome of Acorus Calamus in concentrations of 0.007-0.03g/ml reduced the cells viability in culture after 48 hours of exposure. The most pronounced effect of reducing cell viability was established at the concentration of 0.03 g/ml - 65.93% (p <0.05). Similarly, at concentrations of 0.015 and 0.007g/ml, the research substance led to an increase in the number of dead cells, reducing the viability to 74.29 and 80.24% (p <0.05), respectively. The smallest of the studied concentrations (0.001–0.004 g/ml) did not have any significant toxic effect on Human Hepatocellular Carcinoma cells at the 48 hours of exposure.(Fig. 1.)

The cytotoxicity of the studied solutions increased with the increasing of the time contact up to 72 hours. So, when the cells were incubated with a solution at the lowest concentration (0.001g/ml) for 72 hours, the percentage of dead cells was 16.50% (p <0.05). A further increase of the concentration of the active substance (0.002; 0.004; 0.007; 0.015g/ml) led to a proportional increase in the percentage of dead cells to 19.34; 20.85; 32.06; and 36.75% (p <0.05), respectively. Adding a solution of 0.03g/ml to the culture medium led to the death of 38.42% of the cells (p <0.05).

It should be noted that the increase of the contact time of the studied solutions with HepG2 cells up to 96 hours, the cell survivals of all studied concentrations were significantly lower than at 48 hours, but did not significantly differ from the cell survival at 72 hours, which is probably due to inactivation of the investigated substance.

At the same time, aqueous solutions of the substance obtained from Acorus Calamus Leaves in concentrations of 0.007–0.03g/ml did not significantly affect the cell viability in culture in all studied exposures (Fig. 2), which indicates the absence of toxic effect on the functional activity of mitochondria of the Leaf extract16.

Conclusion

1. The approaches of this study and obtained results have shown that it is possible to evaluate toxicity of extracts of roots and leaves of Sweet Flag (Acorus Calamus) on the culture of Human Hepatocellular Carcinoma in vitro. Which could be used for further research of tolerability and effectiveness of the medicines developed on the basis of the studied substance.
2. The number of dead cells (HepG2) increases from 19.76% to 34.07% (p<0.05) within 48 hours contact with the substance obtained from Sweet Flag (Acorus Calamus) at concentrations 0.007-0.03 g/ml.
3. Cytotoxic effect of the studied substance on the HepG2 cells increases as the time of contact also increases up to 72 hours (p<0.05). But increasing the time to 96 hours didn't show any reduction in cell viability as compared to 72 hours exposure (p<0.05).
4. The water extract of the leaves of Sweet Flag does not affect cell viability at the concentrations of 0.007-0.03 g/ml which indicates the absence of a toxic effect on the functional activities of mitochondria.

The substance obtained from the roots of Sweet Flag could be recommended for further research and development of the anticancer drugs for the treatment of Hepatocellular Carcinoma and other oncological diseases.

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Figure 1. The effect of the substance, obtained from the root of Sweet Flag (REA) , on the viability of human hepatocellular carcinoma cells HepG2, determined in the MTT test, n = 3. * – the differences are significant relatively to the negative control, p<0.05.

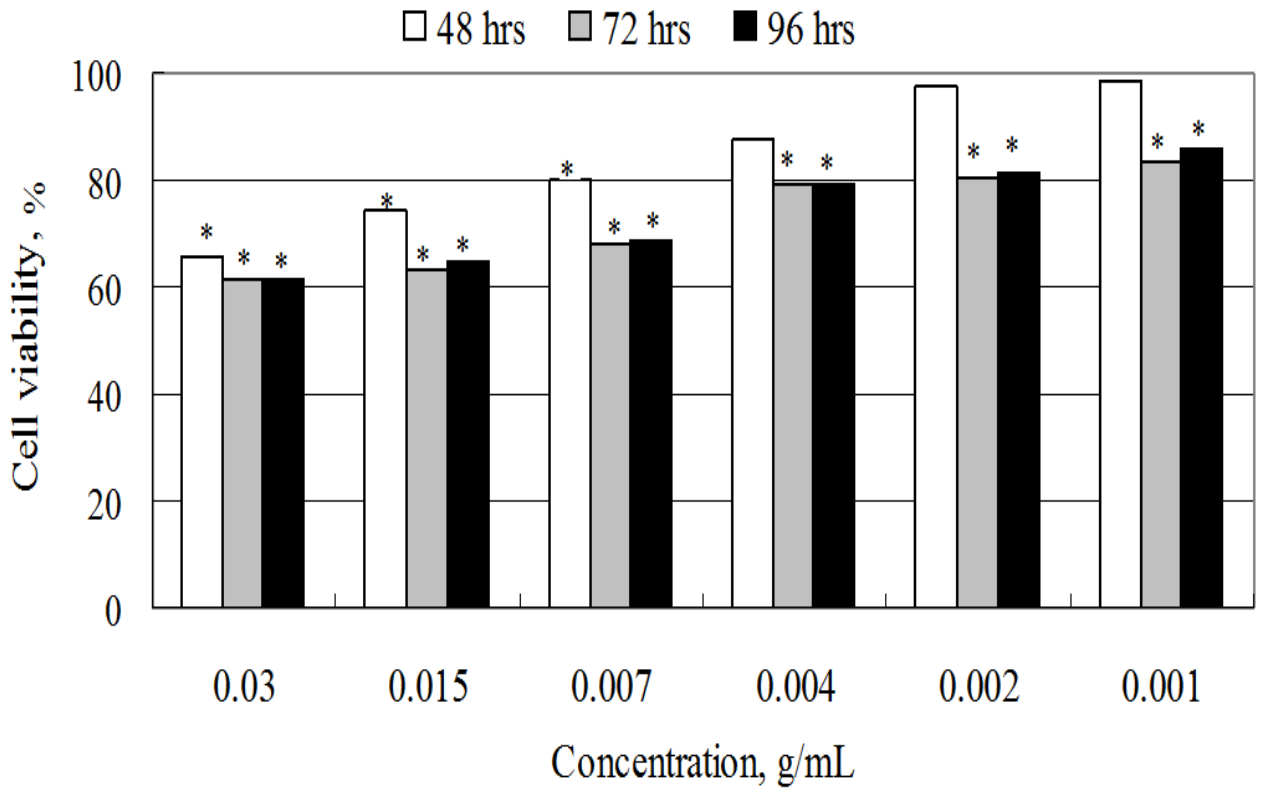

Figure 2. The effect of the substance, obtained from the leaves of Acorus Calamus (LEA), on the Human Hepatocellular Carcinoma (HepG2) cells viability, determined in the MTT test, n = 3.