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Original scientific paper

BIOINFORMATICS ONLINE SUPPORT FOR BIOACTIVE SUBSTANCES CYTOTOXICITY TESTING AND THEIR STATISTICAL ANALYSIS

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ABSTRACT. Preclinical *in vitro*/*in vivo* testing is the first step in discovery of anticancer medicines, among others evaluation of cytotoxic activity of bioactive substances (BAS) on various human normal and cancer cell lines. Cytotoxicity expressed as IC_{50} value (a dose that inhibits 50% of cell growth) is one of the most commonly used parameter for comparable analysis of activity between different substances. This study includes examination of number of BAS and their results for cytotoxic activity obtained in the Laboratory for cell and molecular biology (LCMB) that require various statistical and computational techniques for proper effective analysis. In order to improve experimental data analysis, make it faster, more effective, error proof with secure online data repository, a web application with LCMB IC_{50} database was developed as a useful research tool acting as a leverage for scientific data processing requirements. Analysis includes cytotoxic effects of chemical and natural BAS (IC_{50} values) on HCT-116, SW-480, MDA-MB-231, and MRC-5 cell lines. Generally, it can be concluded that BAS of different origin, chemical or natural, have various cytotoxic effects and cause different cell line sensitivity, which is presented and discussed. This paper presents developed SQL database-centric web application with remote user-friendly data management for a biology researcher user type profile. Data processing in this article can be useful for a further overlook and testing of cytotoxic substances.

Keywords: ANOVA, cancer cell lines, LCMB IC_{50} database, remote access.

INTRODUCTION

Cancer represents one of the most common diseases worldwide. There are many attempts to improve anticancer therapy, where one of the main is using a variety of drugs from different origins and mechanisms of action. All of them must pass through preclinical and clinical testing on diverse model systems (LOPEZ-LAZARO, 2015). The preclinical investigations begin with isolation, purification and/or modification of bioactive substances (BAS)

originating from natural sources, as well as the synthesis of new chemical compounds. They include testing of structural characteristics, their interaction with biomacromolecules and many assays that evaluate their anticancer properties, mainly on the recommended panels of cell lines and animal models. *In vitro* cytotoxic testing of BAS on cancer cell lines allows early detection of cytotoxic treatments that potentially can be used in anticancer therapy (FLORENTO *et al*., 2012). Testing the effect of different BAS on cell viability in culture is a widely used method. There are a large number of *in vitro* cytotoxicity assays to test whether a compound is toxic to cultured cells or not, mainly by determining the number of living cells, over a defined incubation period (RISS *et al.*, 2011). Inhibitory concentration (IC_{50}) of a testing substance that inhibits 50% of cell growth is a referent value for cytotoxicity (CALDWELL *et al*., 2012; ĆURČIĆ *et al*., 2012a). Intensive research in this field, and the large number of results for cytotoxicity obtained from various active substances, and their use in the prevention and treatment of cancer (MILUTINOVIĆ *et al.*, 2015a), lead to statistical processing of these data and implementation of new LCMB IC_{50} database presented in this article.

The anticancer activity of chemical complexes has been known for decades and many of them have been used as a treatment for various types of cancer (NDAGI *et al*., 2017). The impressive effect of cisplatin on cancer cells has launched develop of new derivatives with improved pharmaceutical effects. In recent years, various chemical compounds and metal-based complexes have been increasingly tested, such as platinum, palladium, gold, ruthenium, etc. have been synthesized with different ligands (PETROVIĆ *et al*., 2015; NDAGI *et al*., 2017; ŽIVANOVIĆ *et al*., 2017; RADISAVLJEVIĆ *et al*., 2019; MEDJEDOVIĆ *et al*., 2020).

Natural products isolated from medicinal plants, whether as pure compounds or as extracts, provide limitless opportunities for obtaining new antitumor drugs (CHEN *et al*., 2012). More than 50% of cancer patients in the USA use agents originating from plants or nutrients, either solely or in combination with chemotherapy (WANG *et al*., 2012). Many of plant extracts were recommended as noticeable cytotoxic substances on various cancer cell lines (STANKOVIĆ *et al*., 2011; ĆURČIĆ *et al*., 2012a; MILUTINOVIĆ *et al*., 2015b; MILUTINOVIĆ *et al*., 2019b). The main advantage of BAS from natural origin is cell selectivity, where they affect cancer but not normal, healthy cells (MILUTINOVIĆ *et al*., 2015a; MILUTINOVIĆ *et al*., 2019b). The reported anticancer properties of natural products, including the plant, mushroom and lichen extracts were mainly by induction of apoptosis, modulation of redox status in the cancer cell lines, antimigratory and antiinvasive effects (MILUTINOVIĆ *et al*., 2015a; ŠEKLIĆ *et al*., 2016; CVETKOVIĆ *et al*., 2019; MILUTINOVIĆ *et al*., 2019b). In the Laboratory for cell and molecular biology (LCMB), the cytotoxicity of more than 50 chemically synthesized compounds was examined, as well as over 200 substances from natural origin. Thus, there was the need for collecting IC_{50} data of various BAS sources in the LCMB IC_{50} database for efficient analysis and comparison of cytotoxic effects between different cell lines, as well as within the treatment incubation period, 24 and 72 h. Also, the difference in the cytotoxic effect of BAS isolated from plants, fungi or lichens depending on cell lines and incubation period, as well as for the type of extract used for isolation was further analyzed.

Besides required complex laboratory procedures and tests, which are subject to permanent innovation and improvement, adequate support for large amounts of laboratory data processing becomes increasingly important. Laboratories generate more and more data that need to be adequately processed in order to extract new scientific information and produce new research insights and discoveries. Usual manual data processing supported with various software tools that require significant human assistance like excel tables and similar, cannot cope with increasing data amount, slow and error-prone processing due to required intensive human interaction while having limited data processing and interpretation capabilities and lacking support for complex data processing, decomposition and restructuring. Decomposition and data restructuring (EMELYANOV, 2018) can relate and organize existing data in a new way offering previously unavailable insights and aspects, discovering “hidden” information

which may lead to new scientific achievements, thus giving added value and use of collected laboratory data.

The aims of this study are: (1) collecting IC_{50} values in LCMB IC_{50} database for easy and rapid analysis of given effects BAS cytotoxicity and comparison of cytotoxic effects of different sources of BAS, and (2) developing a web application for remote management of database, containing laboratory data, supporting different modes of operation such as: adaptive filtering for data selection and statistical analysis, parameter and data CRUD (Create Read Update Delete) operations for manipulation of parameter data characterizing IC_{50} values and IC_{50} data edit. All web application operational modes are available remotely, using an intuitive web user interface for a logged-in user.

MATERIALS AND METHODS

Description of Laboratory data and methods

Collected Laboratory data for IC_{50} values are based on the results of the researches performed within the Laboratory for Cell and Molecular Biology, Faculty of Science, University of Kragujevac for the 2010-2020 period. In these researches, 115 different treatments for HCT-116, 56 for SW-480, 62 for MDA-MB-231, and 19 for the MRC-5 cell line were examined. All researches were carried out within the framework of a scientific research project of the Ministry of Education, Science and Technological Development of the Republic of Serbia (Preclinical testing of bioactive substances - PIBAS, III41010).

Human adherent colorectal carcinoma cell line (HCT-116), colorectal adenocarcinoma (SW-480), breast carcinoma cell line (MDA-MB-231), and the human fetal lung fibroblasts-(MRC-5), as normal cell line, were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were cultured in the optimum conditions, according to standard protocols (MILUTINOVIĆ *et al.*, 2019a). The cytotoxicity of various substances on cell lines was examined using the MTT cell viability assay (MOSMANN, 1983). For the purpose of this essay, cells were seeded in 96-well plate, incubated for 24 h and then treated with different substances in a concentration range of 0.1 to 500 μM for chemically synthesized or 0.1 to 500 μg/ml for natural extracts. The assay was performed 24 and 72 h after treatments.

Percentages of cell viability were calculated as the ratio of absorbance of the treated group divided by the absorbance of the control group (untreated cells), multiplied by 100. The IC_{50} value was presented as the parameter for cytotoxicity and comparable result between different substances. The IC_{50} values were calculated from dose curves of cell viability using CalcuSyn v 2.1. software. The IC_{50} values of BAS are grouped according to their origins, such as chemically synthesized (CHS) and natural extracts (NE). The data of natural extracts are grouped according to treatment taxonomy type: plants, fungi, lichens, as well as according to the type of extract used in extraction procedures: methanol, acetone and other types (ethanol, ethyl acetate, water, chloroform and petroleum ether).

Statistical analysis of IC_{50} data

Statistical analysis of IC_{50} data was performed in the web application within the LCMB IC_{50} database presented in this article. The results are presented as mean ± standard error. Analysis of variance (ANOVA) on web application was used as a test to compare selected data. All data that were not read by MTT (values of 0) were excluded from the statistics by filtering and eliminating those values. All data greater than 500 (values >500 μM, and >500 μg/ml) were considered as non-cytotoxic and their values were included in statistics as values of 500.

LCMB IC_{50} database description

Starting point for a database and web application development was a collection of Laboratory data for IC_{50} organized in the form of a single excel table. In the table, columns were IC_{50} laboratory research data and various parameters characterizing experimental conditions of obtained IC_{50} data. Each of more than 360 table lines corresponds to the results of particular lab experiment. Such organization and storage of experimental data offered some basic features for results analysis, while more advanced specific custom analysis and teamwork requirements could not be met with a single table document, even with shared document collaborative environment in the cloud, such as Google disk, Microsoft One drive and other. Such collaborative cloud environments are suitable for teamwork where standard documents like excel, word, and similar are appropriate. Specific analysis features required data decomposition and relational database storage, together with development of an application with custom functionalities. Custom web application for remote management of database containing decomposed laboratory data within safe Faculty's academic network domain was considered as an optimal solution. Figure 1 presents part of the spreadsheet with original Laboratory data.

	A	B	C	D	E	F	G
1	Treatment	IC50 unit	Type of treatment	Cell line	Type of extract	24h	72h
2	2-(phenylselenomethyl)tetrahydrofuran	µM	Chemical compound	HCT-116		>500	>500
3	2-(phenylselenomethyl)tetrahydropyran	µM	Chemical compound	HCT-116		>500	>500
4	5-Fluorouracil	µM	Chemical compound	HCT-116		0.022±0.006	1.35±0.38
5	Allium flavum	µg/ml	Plant	HCT-116	Methanol	28.29 ± 1.24	35.09 ± 0.91
6	Allium flavum	µg/ml	Plant	HCT-116	Ethylacetate	84.76 ± 1.21	35.00 ± 1.39
7	Allium flavum	µg/ml	Plant	HCT-116	Aceton	62.17 ± 1.19	14.80 ± 1.39
8	Allium flavum and 0.1 µM Pd(II) complex (palladium(II) complex with 3-[(2-hy	µM	Cotreatment	HCT-116	Methanol	12.85 ± 0.13	10.51 ± 1.01
9	Allium flavum and 0.1 µM Pd(II) complex (palladium(II) complex with 3-[(2-hy	µM	Cotreatment	HCT-116	Ethylacetate	186.14 ± 3.29	32.21 ± 2.32
10	Allium flavum and 0.1 µM Pd(II) complex (palladium(II) complex with 3-[(2-hy	µM	Cotreatment	HCT-116	Aceton	7.92 ± 0.35	16.84 ± 0.89
11	Allium flavum and 10 µM Pd(II) complex (palladium(II) complex with 3-[(2-hyd	µM	Cotreatment	HCT-116	Methanol	1.60 ± 0.05	1.74 ± 0.08
12	Allium flavum and 10 µM Pd(II) complex (palladium(II) complex with 3-[(2-hyd	µM	Cotreatment	HCT-116	Ethylacetate	0.016 ± 0.001	1.13 ± 0.02
13	Allium flavum and 10 µM Pd(II) complex (palladium(II) complex with 3-[(2-hyd	µM	Cotreatment	HCT-116	Aceton	2.49 ± 0.12	3.74 ± 0.02
14	Au complex - SR06 (dinuclear gold(III) complexes with bidentate ligand 1,4-di	µM	Chemical compound	HCT-116		72,62	29,88
15	Au complex - SR13 (dinuclear gold(III) complexes with bidentate ligand 1,8-di	µM	Chemical compound	HCT-116		60,17	37,16
16	Au complex - SR32 (trinuclear gold(III) complexes with bidentate ligand 1,8-di	µM	Chemical compound	HCT-116		63,34	133,87
17	Au complex - SR33 (trinuclear gold(III) complexes with bidentate ligand 1,6-di	µM	Chemical compound	HCT-116		23,28	24,18
18	Au complex - SR34 (trinuclear gold(III) complexes with bidentate ligand 1,4-di	µM	Chemical compound	HCT-116		0,25	15,88
19	Au complex- SR14 (dinuclear gold(III) complexes with bidentate ligand 1,6-dia	µM	Chemical compound	HCT-116		56,5	52,03
20	Cladonia foliacea	µg/ml	Lichen	HCT-116	Methanol	265.554±13.27	122.474±9.79
21	Coprinus comatus	µg/ml	Fungus	HCT-116	Methanol	>500	>500
22	Cordyceps sinensis	µg/ml	Fungus	HCT-116	Methanol	>500	>500
23	Cisplatin (cis-diamindihloroplatina(II))	µM	Chemical compound	HCT-116		263.66±8.02	104.41±9.01

Figure 1. Original spreadsheet Laboratory data.

Excel data were converted to an equivalent database table. For a web application with required functionalities, data from the obtained table should be decomposed on a number of tables containing data of the same kind corresponding to data in spreadsheet columns. Decomposition is performed programmatically by SQL queries executed from phpMyadmin (https://www.phpmyadmin.net/) database tool or PHP program files (https://www.php.net/). PHP is used as a web server programming language for web applications. Table expdatadecomp is used as the main table joining decomposed data from other tables that are connected with lines in Figure 2 which shows the LCMB IC_{50} database. Other important tables are for instance table sample containing dynamic definition of samples for ANOVA statistic test. Dynamic definition means that only values of parameters defining sample are stored, not sample data, which are dynamically obtained by SQL query when needed, according to the principle of storing only fundamental data and calculating dependent data.

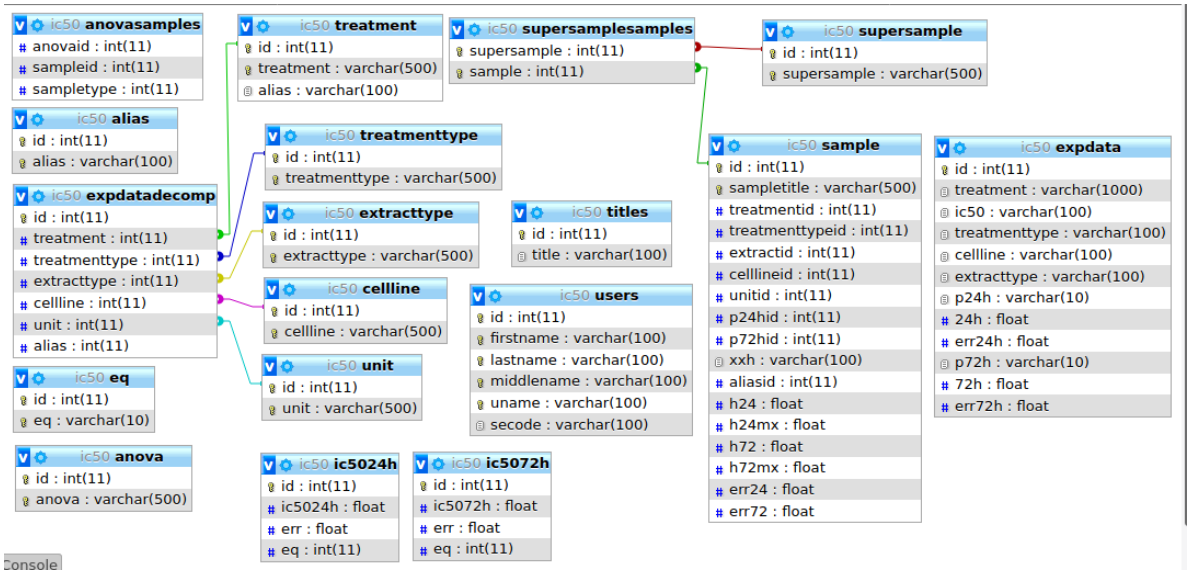


Figure 2. Relational view of LCMB IC_{50} database.

Web application

Web application page for remote access and management of IC_{50} data is presented in Figure 3. There are 3 main functionalities of IC_{50} web application: 1) data view with flexible filtering, 2) data management CRUD operations, and 3) ANOVA statistical analysis including flexible samples creation, single and compound samples. Each of these functionalities includes many other sub functionalities. Any number of filtering conditions can be selected, and only selected parameters actually contribute to filtering. Figure 4 shows the case of data filtering that returns only 2 data rows. Without selection of any filtering parameter, all data are returned, as in Figure 3 where all 360 existing rows are returned. Filtering parameters are: Treatment specifying exact name of the treatment, Alias specifies the name of the treatment more closely, Type specifying type of the treatment, Line for cell line which is used for testing, Unit for IC_{50} and additional filtering parameters. Additional filtering parameters allow specifying 24 or 72 hours interval for applying cytotoxic substance, filtering according to IC_{50} value interval, lower and upper interval value for 24 and 72 h separately, and finally filtering according to IC_{50} error concentration value.

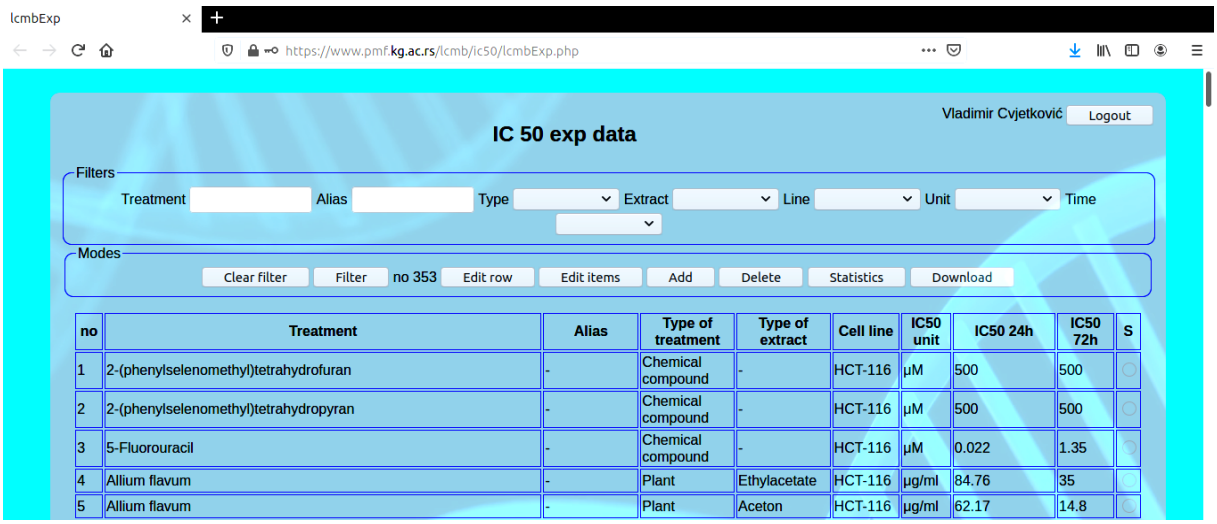
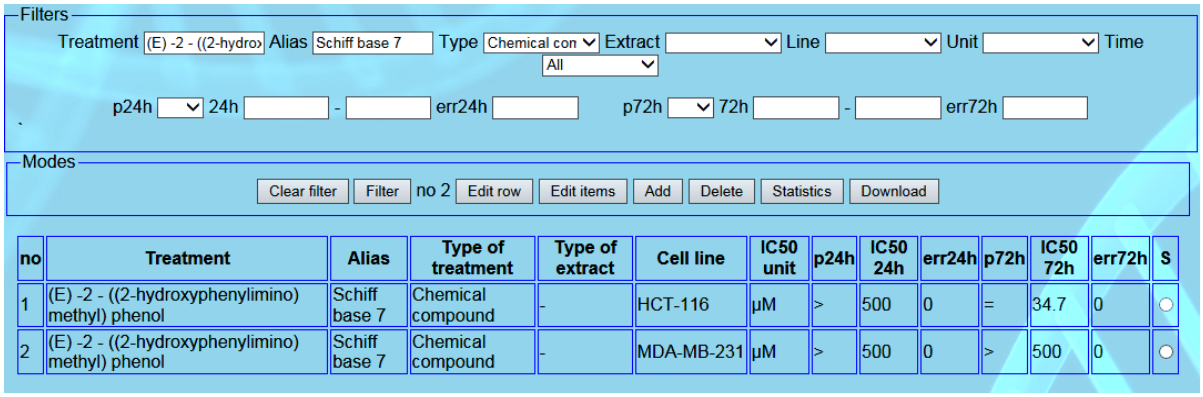


Figure 3. IC_{50} web application page.



no	Treatment	Alias	Type of treatment	Type of extract	Cell line	IC50 unit	p24h	IC50 24h	err24h	p72h	IC50 72h	err72h	S
1	(E) -2 - ((2-hydroxyphenylimino) methyl) phenol	Schiff base 7	Chemical compound	-	HCT-116	µM	>	500	0	=	34.7	0	
2	(E) -2 - ((2-hydroxyphenylimino) methyl) phenol	Schiff base 7	Chemical compound	-	MDA-MB-231	µM	>	500	0	>	500	0	

Figure 4. Data filtering.

Error value is estimated as a maximal value for concentration deviation from given nominal IC_{50} value. Figure 3 shows those additional filtering parameters as text boxes on a web page for entering those values. As for the mentioned filtering parameters, if values are not specified, there is no filtering.

Figure 5 shows web panel for data management CRUD operations. New laboratory experiment data can be added, existing updated, or deleted. Also, new parameter data can be added, updated, or deleted, thus allowing full control of data edit.

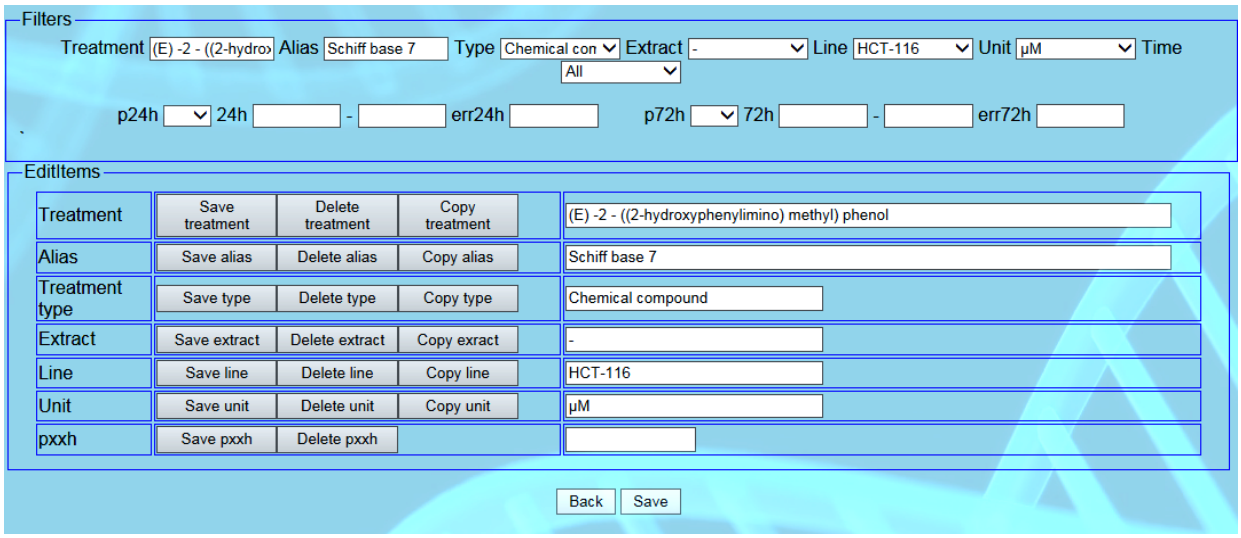


Figure 5. Data management CRUD operations.

Statistical analysis can be performed on any filtered group of IC_{50} data, according to previously described filtering for data view. Figure 6 shows the statistical panel that consists of 3 main subsections: 1) Single sample, 2) Super or compound sample and 3) ANOVA statistics. Simple sample means that a sample for ANOVA test can be defined according to any possible combination of filtering parameters. Sample name is automatically generated from selected filter parameters, with optional sample name prefix and stored in a sample table with selected values of parameters. Two or more simple samples can be compared by ANOVA sample test.

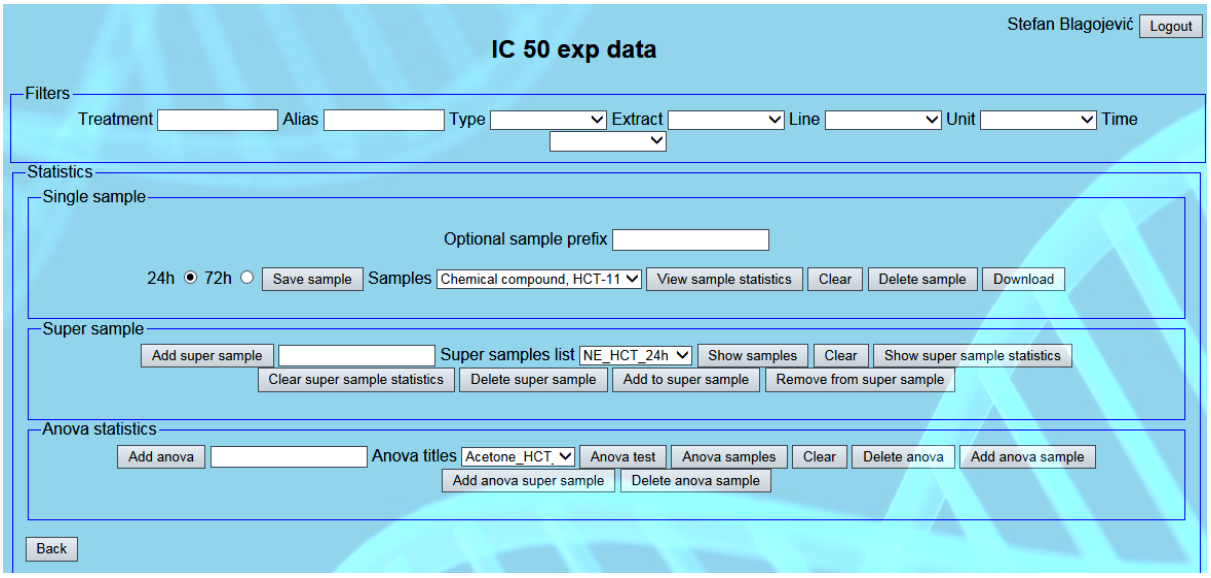


Figure 6. Statistical web application IC_{50} panel.

Figure 7 shows descriptional statistics for selected single sample, sample size 46 which is number of sample data items, and all sample items.

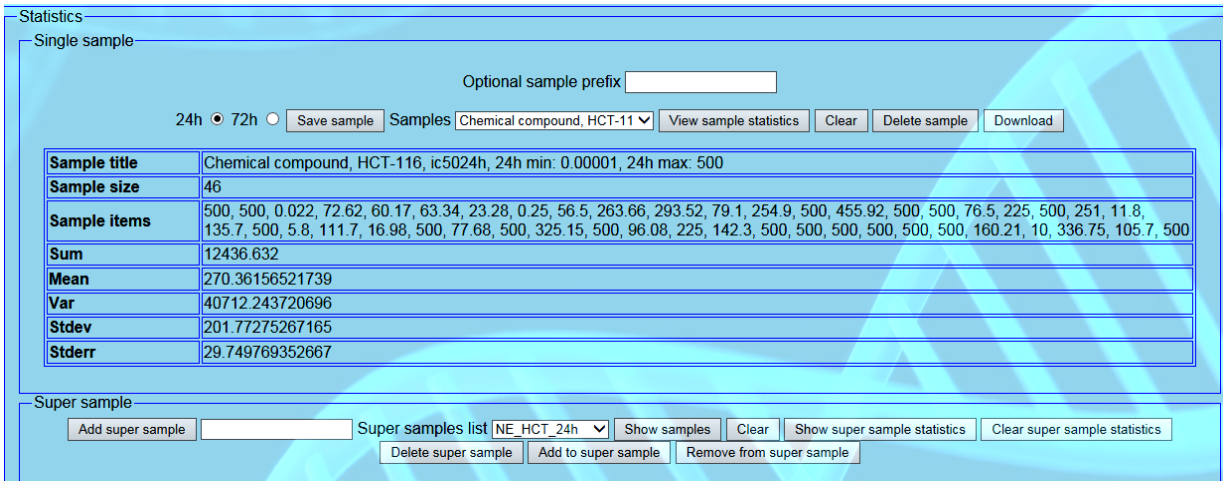


Figure 7. Single sample descriptional statistics.

Figure 8 shows super or compound sample panel. Super sample may consist of one or more previously defined single samples. Super sample consisting of single samples act as one big or compound sample for the ANOVA test. Any combination of two or more single or super samples can be compared with ANOVA test which includes single/single, super/single, or super/super where the number of single/super samples can be one or more. Such samples arrangements offer the flexibility of web application for statistical testing of arbitrary samples. Sample title column in Figure 8 contains titles of all single samples that constitute super sample with title NE_HCT_24h. Each of single samples can be deleted or new single sample added. Similar to the single sample, descriptional statistics for super sample is presented, with a number of super sample data items (No. 69), and all super sample data items listed. Super sample including each single sample is dynamically defined and created from the LCMB IC_{50} database for analysis when needed.

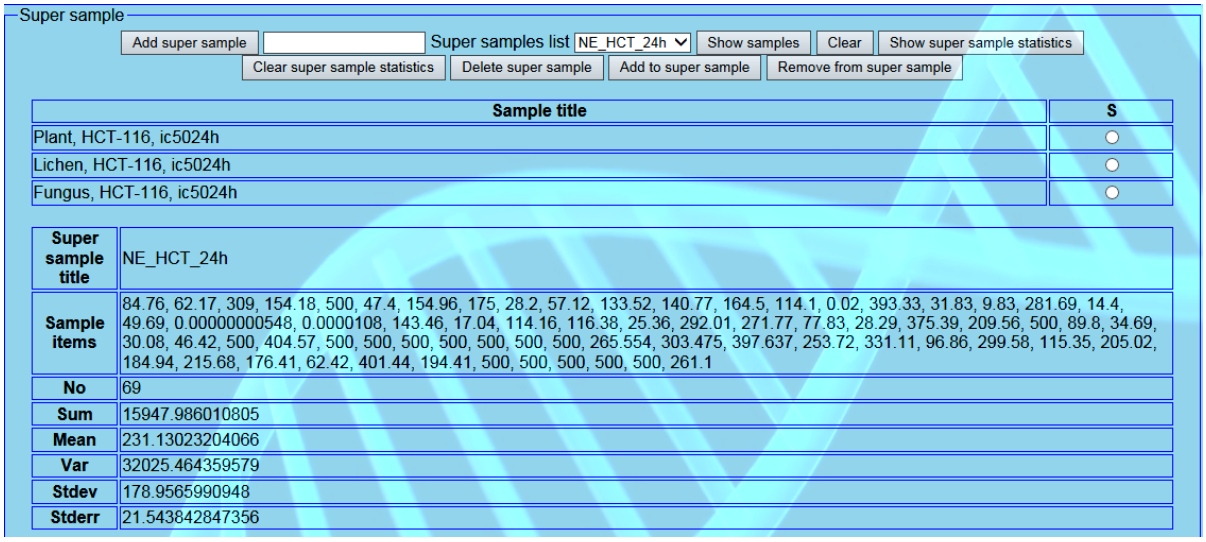


Figure 8. Super sample descriptional statistics.

Figure 9 shows Anova statistics panel. Column Sample title contains single/super sample names for statistical test, 4 samples in this case. In the table below are ANOVA statistical test results. Each of the current samples can be deleted or new samples added to the test.

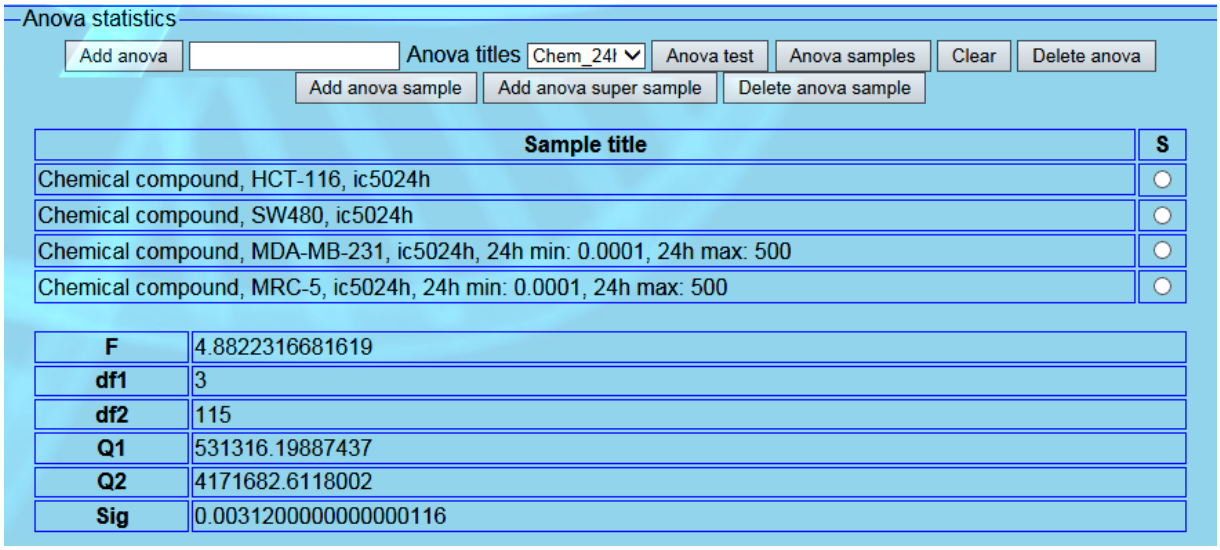
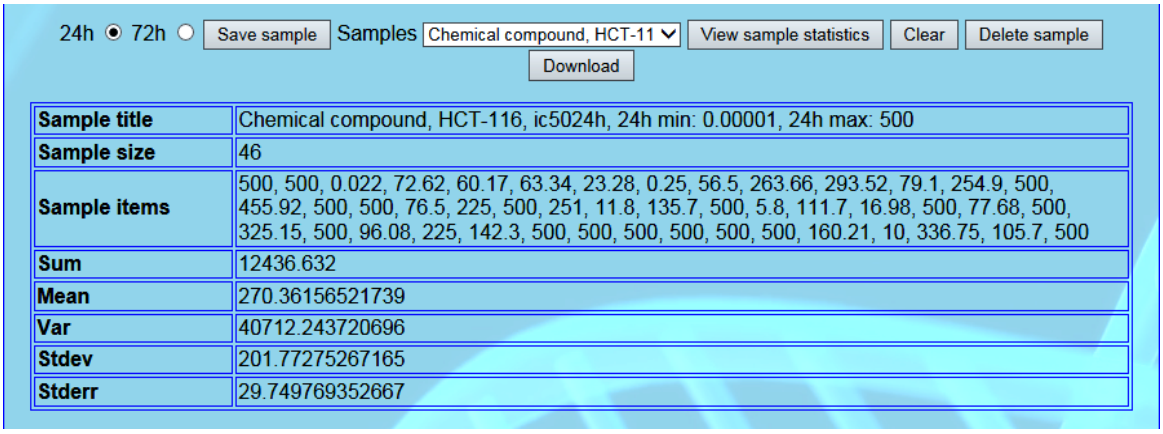


Figure 9. ANOVA statistical test.

RESULTS

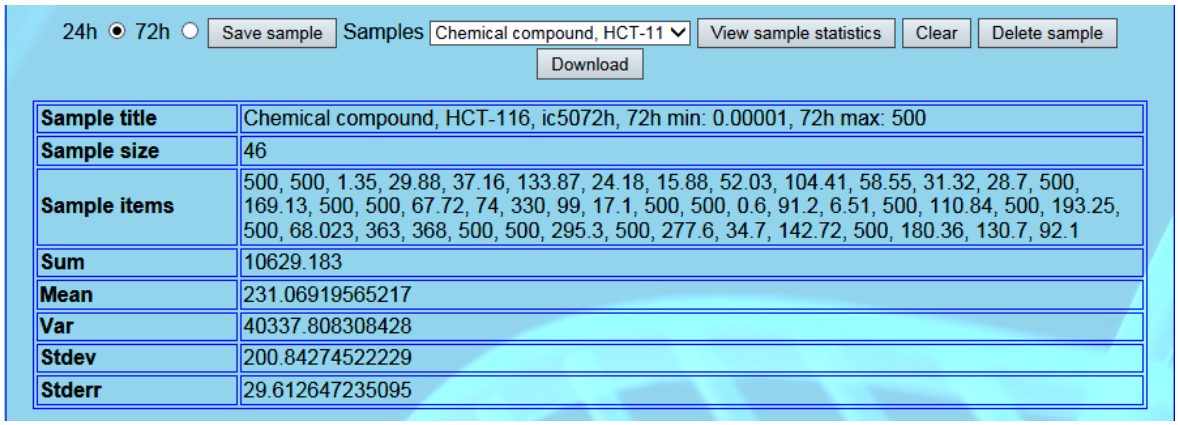
All data for mean, standard error and significance on investigated samples were obtained on the same principle as described in the following example. IC_{50} values for CHS treatments on HCT-116 cell line after 24 h which mean value is presented in Table 1 are obtained from descriptive statistics in web application from LCMB IC_{50} database (Fig. 10). Selected sample with name in Sample title "Chemical compound, HCT-116, ic5024h, 24h min: 0.00001, 24h max: 500" has descriptive statistics with 46 values in Sample size (N). All sample values are listed in Sample items, followed by values for Sum, Mean, Var, Stdev and Stderr. Values for Mean (270.36) and Stderr (29.75) are presented in Table 1 for CHS on HCT-116 cell line after 24 h.



Sample title	Chemical compound, HCT-116, ic5024h, 24h min: 0.00001, 24h max: 500
Sample size	46
Sample items	500, 500, 0.022, 72.62, 60.17, 63.34, 23.28, 0.25, 56.5, 263.66, 293.52, 79.1, 254.9, 500, 455.92, 500, 500, 76.5, 225, 500, 251, 11.8, 135.7, 500, 5.8, 111.7, 16.98, 500, 77.68, 500, 325.15, 500, 96.08, 225, 142.3, 500, 500, 500, 500, 500, 500, 160.21, 10, 336.75, 105.7, 500
Sum	12436.632
Mean	270.36156521739
Var	40712.243720696
Stdev	201.77275267165
Stderr	29.749769352667

Figure 10. Descriptive statistics for CHS on HCT-116 cell line after 24 h.

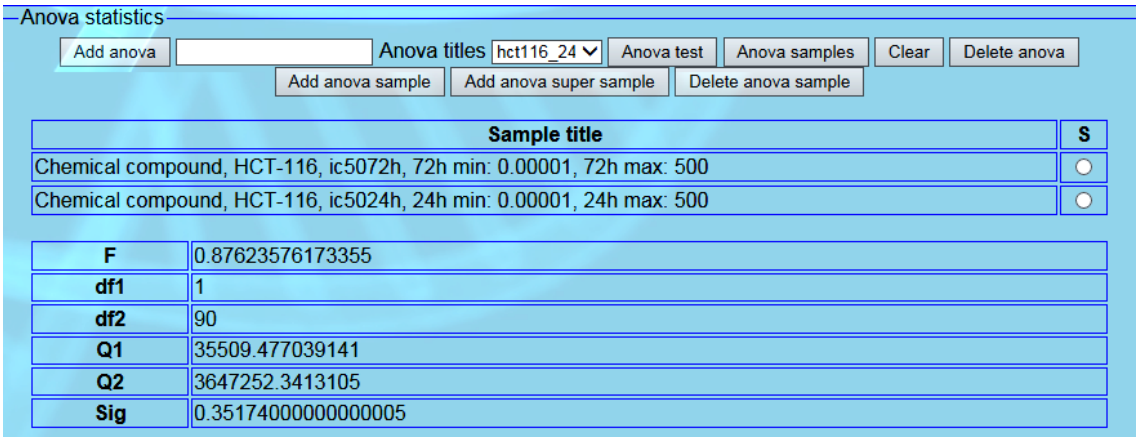
Values for CHS on HCT-116 cell line after 72 h, are obtained from descriptive statistics for sample with Sample title "Chemical compound, HCT-116, ic5072h, 72h min: 0.00001, 72h max: 500" in Figure 11. Sample size of 46, mean value of 231.07 and standard error of 29.61 are presented in Table 1.



Sample title	Chemical compound, HCT-116, ic5072h, 72h min: 0.00001, 72h max: 500
Sample size	46
Sample items	500, 500, 1.35, 29.88, 37.16, 133.87, 24.18, 15.88, 52.03, 104.41, 58.55, 31.32, 28.7, 500, 169.13, 500, 500, 67.72, 74, 330, 99, 17.1, 500, 500, 0.6, 91.2, 6.51, 500, 110.84, 500, 193.25, 500, 68.023, 363, 368, 500, 500, 295.3, 500, 277.6, 34.7, 142.72, 500, 180.36, 130.7, 92.1
Sum	10629.183
Mean	231.06919565217
Var	40337.808308428
Stdev	200.84274522229
Stderr	29.612647235095

Figure 11. Descriptive statistics for CHS on HCT-116 after 72h.

The p value of 0.3517 is obtained from ANOVA test named hct116_24_72 which compares previous two samples for CHS on HCT-116 after 24 and 72 h (Fig. 12). Column Sample title contains names of all tested samples (previously mentioned and discussed), with obtained Sig (significance) value of 0.3517, which is p value in Table 1.



F	0.87623576173355
df1	1
df2	90
Q1	35509.477039141
Q2	3647252.3413105
Sig	0.35174000000000005

Figure 12. ANOVA test for selected samples.

ANOVA test between cell lines for 24 h, is presented in Figure 13. It can be seen that four samples are compared, for each cell line. Obtained Sig is 0.0031 which is p value for CHS between cell lines after 24 h (Tab. 1).

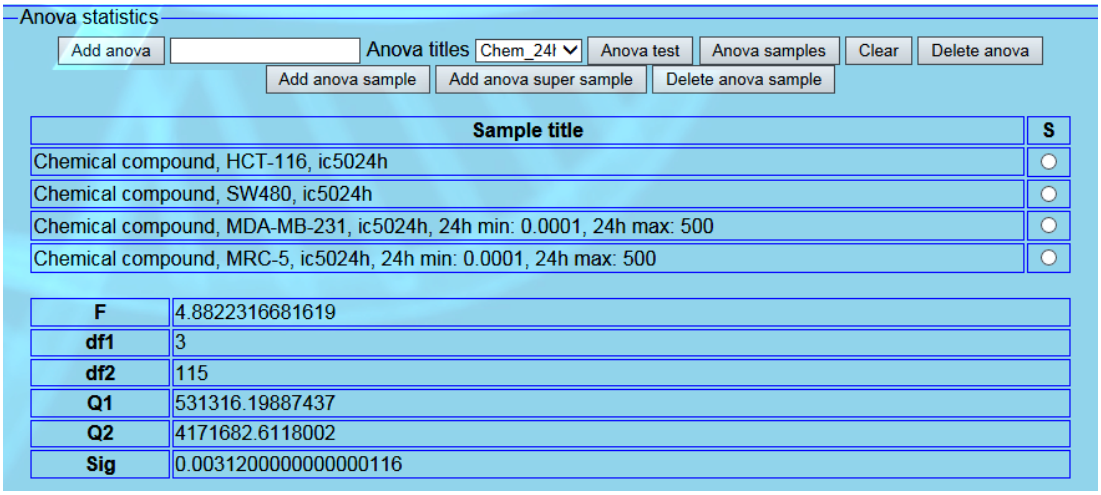


Figure 13. ANOVA test for CHS treatments between cell lines after 24 h.

Analysis of variance for cytotoxicity of BAS was performed depending on the unit in which IC_{50} values are expressed. The IC_{50} values differ to their origin: isolated from natural sources (plants, lichen and fungi) expressed in μg/ml and chemically synthesized expressed in μM, which is why the statistical significance was not examined between this two samples. Basic descriptive statistics and values for significance (p) obtained by the ANOVA test from previously presented database, are shown in Table 1. Analysis of variance shows that there is a statistically significant difference in the cytotoxicity of CHS ($p = 0.0031$) treatments after 24 h between different cell lines. MRC-5 cell line is the most sensitive in response to CHS treatments and the most resistant to NE treatments. MDA-MB-231 is more resistant to CHS treatments compare to the other cell lines. Analysis of variance for cytotoxicity data of CHS and NE treatments after 72 h compared to cell lines shows that there is no significant difference. These results indicate a similar sensitivity of the cell lines within the selected treatment conditions after longer incubation time. Table 1 shows that there is no significant difference within cell lines compared to the incubation time of 24 and 72 h, indicating that the treatment acts cytotoxic, constantly during the selected treatment periods. The exception is MDA-MB-231 cells, where CHS treatments show stronger cytotoxic activity over time.

Table 1. Basic descriptive statistics and variance analysis values for IC_{50} values of CHS (μM) and NE (μg/ml) in relation to cancer cell type after 24 and 72 h.

CHS	Cell line				p^a	N
	HCT-116	SW-480	MDA-MB-231	MRC-5		
24 h	270.36±29.75	307.34±48.80	354.12±27.92	137.73±44.13	0.0031*	46/16/42/14
72 h	231.07±29.61	248.20±54.68	262.11±30.74	119.42±40.53	0.1244	46/16/44/15
p^b	0.3517	0.4260	0.0299*	0.7619	-	-
NE						
24 h	231.13±21.54	231.42±32.69	309.33±43.50	356.19±35.58	0.0598	69/40/18/14
72 h	179.25±21.32	191.28±30.46	246.42±51.87	311.22±43.45	0.0766	69/40/18/14
p^b	0.0892	0.3719	0.3593	0.4251	-	-

The results are present as mean ± standard error; p^a - statistical difference between cell lines; p^b - statistical difference between 24 and 72 h; *statistically significant difference ($p<0.05$).

Analysis of variance shows that there is a statistically significant difference between source of NE (plants, fungi, lichens) on HCT-116 cell line after 24 ($p = 0.0087$) and 72 h ($p = 0.0019$) of exposure (Tab. 2). These results indicate that plants show the best cytotoxic effect on HCT-116 cell line after 24 h and after 72 h of incubation time. Table 2 shows that there is no significant difference in tested treatment for SW-480 cell line after 24 and 72 h. Mean values show slightly stronger cytotoxic activity of plants after 24 and lichens after 72 h, indicating the different time-dependent effects of these types of treatment on SW-480 cells. There is no statistically significant difference in all tested groups between 24 and 72 h incubation time.

Table 2. Basic descriptive statistics and analysis of variance for NE (μg/ml) IC_{50} values in treatment of HCT-116, SW-480, MDA-MB-231 and MRC-5 cells after 24 and 72 h in relation to the source of NE: Plants, Fungi and Lichens.

Cell line	h	Source of natural extracts			p^a	N
		Plants	Fungi	Lichens		
HCT-116	**24**	201.74±26.87	452.22±47.78	250.23±29.73	0.0087*	48/5/16
HCT-116	**72**	155.27±23.95	440.12±59.88	169.68±40.67	0.0019*	48/5/16
p^b		0.1999	0.8784	0.1203	-	-
SW-480	**24**	210.45±38.28	369.10±80.16	218.04±89.46	0.2876	29/5/6
SW-480	**72**	174.01±37.89	351.20±74.20	141.50±26.21	0.1296	29/5/6
p^b		0.5015	0.8739	0.4308	-	-
MDA-MB-231	**24**	309.33±43.50	-	-	-	18/-/-
MDA-MB-231	**72**	246.42±51.87	-	-	-	18/-/-
p^b		0.3593	-	-	-	-
MRC-5	**24**	413.22±46.37	-	280.17±40.54		8/-/6
MRC-5	**72**	317.65±72.12	-	302.64±35.69	-	8/-/6
p^b		0.2838	-	0.6862	-	-

The results are present as mean ± standard error; p^a – statistical difference between source of natural extracts; p^b – statistical difference between 24 and 72 h; *statistically significant difference ($p<0.05$).

Table 3 presents basic descriptive statistics and analysis of variance for NE IC_{50} in treatment of HCT-116, SW-480, MDA-MB-231 and MRC-5 cells after 24 and 72 h in relation to the type of extracts (acetone, methanol and others) used for extraction of BAS. Based on results for significance between investigated type of extracts it can be concluded that all extracts generally adequately isolate the BAS and to exhibit similar activity on HCT-116 cell line, since no significant difference in cytotoxicity was shown depending on the type of extract. Analysis of variance shows that there is a statistically significant difference of NE IC_{50} values in type of extracts (acetone, methanol and others) on SW-480 cell line after 24 ($p = 0.0148$) of exposure (Tab. 3). Acetone extracts show stronger cytotoxicity on SW-480 cells than others. There is no statistically significant difference in all tested groups between 24 and 72 h incubation time.

Table 3. Values for basic descriptive statistics and analysis of variance for NE (μg/ml) IC_{50} values in treatment of HCT-116, SW-480, MDA-MB-231 and MRC-5 cells after 24 and 72 h in relation to the type of extracts: Acetone, Methanol and Others.

Cell line	h	Type of extracts			p^a	N
		Acetone	Methanol	Others		
HCT-116	**24**	228.73±56.48	236.01±28.42	223.10±42.23	0.9652	9/39/21
HCT-116	**72**	108.02±50.08	194.45±28.02	181.56±41.79	0.4235	9/39/21
p^b		0.1293	0.3010	0.4885	-	-
SW-480	**24**	82.92±26.43	218.11±47.11	342.25±58.25	0.0148*	8/19/13
SW-480	**72**	68.48±32.61	192.69±42.81	264.81±60.47	0.0730	8/19/13
p^b		0.7361	0.6920	0.3655	-	-
MDA-MB-231	**24**	78.49	346.26±44.08	213.96±144.70	-	1/14/3
MDA-MB-231	**72**	8.11	278.01±56.63	178.43±160.81	-	1/14/3
p^b		-	0.3504	-	-	-
MRC-5	**24**	235.90±10.02	394.68±41.06	284.05±110.25	-	2/10/2
MRC-5	**72**	291.16±89.76	330.21±57.52	236.30±19.70	-	2/10/2
p^b		-	0.3737	-	-	-

The results are present as mean ± standard error; p^a – statistical difference between type of extracts; p^b – statistical difference between 24 and 72 h; *statistically significant difference ($p<0.05$).

DISCUSSION

There is a very limited number of studies that analyze and group the database for the cytotoxic effect of bioactive substances. In the following, in order to examine the different effects of cytotoxicity, we discuss whether and why there are significant differences in the action of BAS of chemically synthesized and natural extracts on HCT-116, SW-480, MDA-MB-231 and MRC-5 cell lines, as well as within the treatment incubation period after 24 and 72 h. In addition, results were discussed to investigate the difference in the cytotoxic effect of BAS isolated from plants, fungi or lichens depending on cell lines and incubation period, as well as for the type of extract by which they were isolated.

Based on the results obtained for cytotoxicity of BAS (CHS and NE) to the type of cancer cells treated: HCT-116, SW-480, MDA-MB-231 and MRC-5, it can be concluded that the cell lines are generally stable, because of a large number of data with different values that are included in the assay, as indicated by the standard error. Mean values for IC_{50} indicate that the MDA-MB-231 cell line is partially more resistant compared to HCT-116, SW-480 and MRC-5, however, this difference is statistically significant only for CHS treatments after 24 h. These cell lines do not originate from the same organ which may lead to different sensitivity to the investigated CHS and NE treatments as a consequence of activation of different signaling pathways and mechanisms of cytotoxicity. Besides, the MDA-MB-231 cell line is metastatic and has highly invasive potential, therefore it can be more resistant to the effects of BAS (AMARO *et al*., 2016).

Cytotoxic activity of newly synthesized chemical compounds, potential antitumor drugs was investigated based on published results (ŠMIT *et al*., 2013; JEVTIĆ *et al*., 2014; KOŠARIĆ *et al*., 2014; PETROVIĆ *et al*., 2014; STOJKOVIĆ *et al*. 2014; PETROVIĆ *et al*., 2015; ŽIVANOVIĆ *et al*., 2017; ŠEKLIĆ, 2018; VUKIĆ *et al*., 2018b; VUKOVIĆ *et al*., 2018; RADISAVLJEVIĆ *et al*., 2019; SOLDATOVIĆ *et al*., 2019; MEDJEDOVIĆ *et al*., 2020). One of the most commonly used cytostatic in cancer therapy is cisplatin. Cisplatin achieves its antitumor properties by binding to DNA bases of rapidly proliferating cells, but also interacts with

healthy cells, leading to nephrotoxicity and other adverse effects (STOJKOVIĆ *et al*. 2014). Due to numerous side effects, there are attempts to synthesize novel chemical agents with easier passage through the cell membrane and the cell itself, as well as easier binding to DNA (FLOREA and BUSSELBERG, 2011). The results of the ANOVA test showed that there was a statistically significant difference between the variable of the tested groups in the effect of treatment with CHS after a 24 h incubation period between HCT-116, SW-480, MDA-MB-231 and MRC-5 cells. Comparing the mean values for IC_{50} within the tested cell lines higher viability of MDA-MB-231 cells was observed. MDA-MB-231 cell line was proved to be the most resistant on CHS treatment after 24 h, but after longer exposure of 72 h viability of these cells decreases significantly. Considering the mean values for IC_{50} in chemical treatments, a stronger cytotoxic effect was observed after 72 h compared to 24 in all cell lines. This can be explained by the time-dependent effect that chemical treatments have on cancer cells, that is, they also act antiproliferative.

The cytotoxicity results of natural origin (NE) treatments were processed based on published results (MITROVIĆ *et al*., 2011; STANKOVIĆ *et al*., 2011; ĆURČIĆ *et al*., 2012a; ĆURČIĆ *et al*., 2012b; STANKOVIĆ *et al*., 2012; GRBOVIĆ *et al*., 2013; ĆURČIĆ, 2014; KOSANIĆ *et al*., 2014; ALIMPIĆ *et al*., 2015; MILUTINOVIĆ *et al*., 2015a; MILUTINOVIĆ *et al* 2015b; NIKODIJEVIĆ *et al*., 2016; ŠEKLIĆ *et al*., 20l6; ALIMPIĆ *et al*., 2017a; ALIMPIĆ *et al*., 2017b; DULETIĆ-LAUŠEVIĆ *et al*., 2018; ŠEKLIĆ *et al*., 2018; VUKIĆ *et al*., 2018a; VUKIĆ *et al*., 2018b; CVETKOVIĆ *et al*., 2019; MILUTINOVIĆ *et al*., 2019b; MILUTINOVIĆ *et al*., 2019c; GRUJIČIĆ *et al*., 2020). The results of the ANOVA test showed that there was no statistically significant difference between the mean values of the dependent variable of the test groups in the effect of NE treatment after 24 and 72 h incubation period between HCT-116, SW-480, MDA-MB-231 and MRC-5 cells. By comparing the cell lines with each other, a slightly higher sensitivity is observed on colon cancer cells HCT-116 and SW-480, while MRC-5 cell line proves to be the most resistant to treatment of natural origin. These results can be linked with the less-toxic effects of natural-source treatments on healthy MRC-5 cells compared to chemicals (GREENWELL and RAHMAN, 2015).

Based on the results obtained for cytotoxicity of BAS of natural origin (NE) in the treatments of SW-480 cells after 24 and 72 h relative to their source: plants, fungi, lichens, it can be concluded that the cell lines are generally stable and do not differ significantly in sensitivity to treatments. Treatments of natural origin, categorized as plants, fungal and lichen extracts differ in the type of BAS they contain and may, therefore, exhibit similar or different effects of cytotoxicity on different cell lines (MITROVIĆ *et al*, 2011; ĆURČIĆ, 2014; ŠEKLIĆ *et al*., 2016). It was shown that there was a statistically significant difference for cytotoxicity of BAS of natural origin on HCT-116 cells after 24 and 72 h compared to the source of NE: plants, fungi, lichens. The results of the analysis of variance within these data groups showed that there were no statistically significant differences in the effect of treatment on SW-480 cells for both incubation periods tested. Mushroom-derived treatments were found to be the weakest, while plant and lichen treatments had a slightly stronger and similar cytotoxic effect. The cells of the fruiting bodies of the fungi are rich in sugar components such as glucose, mannose, galactose which can have a beneficial effect on cell proliferation (DOERING *et al*., 2017). Bioactive substances isolated from different natural sources can activate different signaling pathways and mechanisms of cytotoxicity in cancer cell lines. Given the good cytotoxic effect of lichen, an increasing number of different species have been tested over the past few years (MORIANO, 2016).

Based on the results obtained for cytotoxicity of BAS of NE in treatments on HCT-116 cells after 24 h between the type of extract: methanol, acetone and others, it can be concluded that all the extracts generally adequately isolate the BAS that exhibit similar activity. Comparing the mean values for IC_{50} on HCT-116 cells over a 72 h a stronger effect is shown by acetone extract compared to methanolic and others. For SW-480 cells over a 24

and 72 h incubation period, a better effect of cytotoxicity was shown by acetone extract compared to methanolic and other extracts. GUPTA *et al*. (2012) shown that acetone and methanol have been the most effective solvents for the isolation of BAS such as flavonoids and others. Considering the results shown above it can be assumed that acetone extracts additional compounds that can give better effects on SW-480 cells. All types of extracts show time-dependent cytotoxicity on HCT-116, SW-480, MDA-MB-231 and MRC-5 cells.

CONCLUSION

In this study, newly created LCMB IC_{50} database provides a useful way for storage and comparison of results by different categories: related to the origin of bioactive substances, testing cell line, the origin of cell line, period of incubation, etc. Using the LCMB IC_{50} database, results were processed and presented in a new way. This kind of data processing has proven to be useful for a further overlook of bases with cytotoxic substances and it can be helpful in the selection and preparation of new BAS, as well as for prediction of their effect in future investigation. Also, database offers the possibility for highlights and selects the BAS with noticeable cytotoxic activity, support for detailed analysis, developing effective anticancer substance and more noticeable selectivity of BAS against one type of cells, for example, cancer vs normal. Statistical data processing of previously observed results can predict the most applicable model system, cell line for chosen treatment, the most applicable cell line for investigation cancer cell resistance to the treatment after a longer time of exposure and response to which type of extract was more effective for natural substances. Using web application for statistical analysis significantly improved not only the speed of analysis but also eliminated potential errors in defining samples and compound samples once they were selected and added to ANOVA statistical test. Simultaneous use of web application by many researchers is possibly contributing to research work efficiency, making it time and place independent for research results analysis. Given that the LCMB IC_{50} database is currently restricted to log-in users only and contains data obtained from the LCMB, this database has the potential to become a global platform for depositing and accessing such results in the future updates, while meeting the requirements for adequate data protection.

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