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Original Research

In vitro evaluation of the cytotoxicity of Nutri-pepper enhancer in human-like kidney and hepatic cells and mouse fibroblasts

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Abstract

The aim of this paper was to evaluate the cytotoxicity of Nutri-pepper enhancer in fibroblasts, hepatic and kidney cells by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results showed no significant cytotoxic effects of Nutri-pepper enhancer on fibroblasts, hepatic and kidney cells at a concentration range of 0.9375–60 µg/ml.

The IC_{50} values of the sample against the tested cell lines ranged between 5.53–14.39 µg/ml, which are much higher than those for the positive control with the IC_{50} values ranging between 0.0238–0.1179 µg/ml.

This finding indicates that Nutri-pepper enhancer is not cytotoxic to fibroblast, kidney and hepatic cells.

Keywords: Cytotoxicity, Nutri-pepper enhancer, MTT assay, IC_{50}

Introduction

The phytochemical investigations of *Piper nigrum* have revealed that this crop contains a variety of phytochemicals [1] with medicinal properties. Piperine was considered the major active compound with diverse pharmacological activities. Many researchers have reported that pepper extracts contain various types of active ingredients with medicinal properties; for example, phenolics, flavonoids, alkaloids, amides and steroids, lignans, terpenes, chalcones and others [2]. Even though many studies have proven that this plant contains various types of antioxidants with anti-inflammatory, anticancer, antiperiodic and antipyretic properties, the number of nutraceuticals that have been developed from it is still low, which means that this plant has not been fully utilized and exploited.

Solely using pepper extracts as medical herbs reduces the therapeutic effect due to limited bioactive compound content. Since pepper extracts have been reported to contain piperine compounds and can act as food enhancers to increase the absorption of these insoluble active compounds in the human body [3], incorporation of potential active compound extracts from a local underutilized crop with pepper extracts enhances the efficiency, resulting in good nutraceutical products and functional foods.

Nutri-pepper enhancer is a newly developed nutraceutical product containing various types of bioactive compounds including piperine, curcumin and leonurine. A preliminary report showed that this product not only exhibited positive antidiabetic effects, but also demonstrated good anticholesterol and anticancer effects. The biological compatibility of these materials is of vital importance to avoid or limit cell or tissue irritation or degeneration. Most of the biomaterials may have low, medium or high potential risk to human safety, depending on the type and the extent of patient contact. Therefore, one of the ISO standards (ISO 10993-1:2003) [4] was developed and it recommended that appropriate steps should be taken for biological assessment of medical compounds *in vitro* for the determination of any cytotoxic effects of these new biomaterials. In this primary screening, we aimed to evaluate the cytotoxicity of Nutri-pepper enhancer in human and non-human cell lines. Cytotoxicity screening assays provide a measure of cell death caused by materials or their extracts [5]. Primary cell cultures derived from kidney, hepatic and fibroblast cells were used in this study due to their high sensitivity to toxic substances. As the rationale for the development of new therapeutic materials is to enhance successful clinical applications, trials must be carried out to evaluate their cytotoxicity. Hence, the aim of this study was to evaluate the cytotoxicity of Nutri-pepper enhancer on cultured human-like kidney and hepatic cells and mouse fibroblasts.

Objective

The objective of this study was to evaluate the cytotoxicity of Nutri-pepper enhancer in cultured kidney, hepatic and fibroblast cells using the MTT method.

Materials and methods

Test system

Quantification of cell viability and proliferation in response to external factors forms the basis of numerous *in vitro* assays.

An MTT assay is a colourimetric assay based on assessing cell metabolic activity.

Human kidney-like cells (Vero), hepatic cells (WRL-68) and a mouse fibroblast cell line (BALB/3T3 clone A31) were used to assess the cytotoxic potential of Nutri-pepper enhancer *via* initial screening of apoptosis or necrosis.

The biochemical mechanism underpinning the MTT assay involves cellular NAD(P)H-dependent oxidoreductase converting the yellow tetrazolium MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into insoluble (*E,Z*)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan). The formed formazan can be dissolved with dimethyl sulfoxide (DMSO) to give a purple colour with a characteristic absorption at 570 nm. The intensity of the purple colour is directly proportional to the cell number, thus indicating cell viability.

Sample preparation

Nutri-pepper enhancer was obtained from the Malaysian Pepper Board and contained three major components: turmeric extract (curcumin), black pepper extract (piperine) and *Leonurus japonicus* extract (leonurine). The sample stock solutions were prepared by adding DMSO before being stored at -20°C. The different concentrations of Nutri-pepper enhancer (0.9375 to 60 µg/ml) used in this study were prepared from the stock solution along with the chemotherapeutic agent paclitaxel (Taxol), with concentrations ranging from 0.00001 to 1.0 µg/ml.

Cell lines

Human kidney-like cells (Vero), hepatic cells (WRL-68) and mouse fibroblasts (BALB/3T3 clone A31) were purchased from Sigma-Aldrich. Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, Korea) supplemented with 10% foetal bovine serum (FBS; Welgene) and 1X penicillin/streptomycin (P/S; Welgene). Cells were routinely incubated under humidified atmosphere containing 5% CO_2 at 37°C and subcultured when 85% confluent.

Cell line preparation for assays

Cells were plated on 96-well plates (5×10^4 cells/well) for seeding. After 24 hours of cell seeding, a partial monolayer was formed and the optimum cell confluence of 70% was achieved. The 96-well microtitre plates were treated with different concentrations of the sample extract. Approximately 0.1 ml of well-mixed sample extract solution (0.9375 to 60 µg/ml) was added to the 96-well plates. Therefore, the final sample extract concentration was two-fold diluted, ranging from 0.4688 to 30 µg/ml (this dilution factor was taken into account when assessing the results).

The negative control was set as treating the cells with 0.9% saline. Minimum essential medium was included as the blank control.

All of the sample concentrations were prepared in triplicate. The plates were incubated at 37°C for 72 hours before the cytotoxicity test was carried out.

Cytotoxicity assay

An MTT cytotoxicity assay was carried out according to a slight modification of the procedure reported by Mosmann (1983) [6].

At the end of the 72-hour incubation period, the medium in each well was removed, and 50 µl of MTT solution was added. The plates were gently shaken and incubated for 4 hours at 37°C in a 5% CO_2 incubator. The medium with MTT was then removed from the wells.

The remaining formazan was dissolved by adding 0.2 ml of DMSO. The solution was pipetted up and down to dissolve the crystals. An enzyme-linked immunosorbent assay plate reader (Biotek, United States) was used to determine the absorbance at 570 nm with a reference wavelength of 630 nm.

The percentage growth inhibition was calculated using the following formula:

$$\% \text{ cell inhibition} = 100 - [(At - Ab)/(Ac - Ab)] \times 100$$

where

At = absorbance value of the test compound;

Ab = absorbance value of the blank;

Ac = absorbance value of the control.

The percentage cell inhibition against the concentration of the test compounds was plotted. The half maximal inhibitory concentration (IC50) was calculated based on the equation in the plotted graph.

Statistical analysis

All of the data shown are presented as the mean ± standard error of the mean (SEM). Data were obtained from three replications (n=3). Statistical analysis was carried out using Student's unpaired t test and one-way ANOVA.

Results

To determine the effects of Nutri-pepper enhancer on the viability of various cell types (Vero, WRL-68 and BALB/3T3 clone A31), the MTT assay was used to evaluate the metabolic activity of living cells. The cell lines were incubated with different concentrations of Nutri-pepper enhancer extract (0–60 µg/ml). Cell viability was determined after 24 hours of incubation.

As can be seen in **Table 1**, some of the extracts induced cytotoxicity at certain concentrations. For all cell types, Nutri-pepper enhancer induced cytotoxicity in a largely concentration-dependent manner.

Cell viability was considerably reduced at Nutri-pepper enhancer concentrations of 0.9375 to 60 µg/ml. The results obtained show that at a concentration of 15 µg/ml, the Nutri-pepper enhancer sample reduced cell viability below 40% (**Table 1**).

The results also show that the Nutri-pepper enhancer had the strongest cytotoxic effects on fibroblast cells (BALB/3T3 clone A31) with cell viability at 19.92% at 7.5 µg/ml, compared to Vero and WRL-68 cells with a cell viability of 23.21% and 77.0%, respectively, at the same concentration of sample.

The WRL- 68 cell line only showed a cell viability of less than 50% at the concentration of 15 µg/ml, indicating that this extract is less toxic towards hepatic cells. Conversely, the viability of all tested cell lines tended to decrease with increased concentrations of Taxol, a positive control and a commercially available drug used for cancer treatment. At a concentration of 1.0 µg/ml, the Taxol sample had a cell viability of below 50% (**Table 2**).

The 50% maximal inhibitory concentration (IC_{50}) values for Nutri-pepper enhancer with respect to Vero, WRL-68 and BALB/3T3 clone A31 cells were determined by probit analysis using the Pharm/PCS (Pharmacologic Calculation Sys-

Table 1 The survival of Vero, WRL-68 and BALB/3T3 clone A31 cell lines after 24 hours of treatment with Nutri-pepper enhancer

Nutri-pepper enhancer (µg/ml)	Cell type (% viability)					
	Vero		WRL-68		BALB/3T3 clone A31	
	Mean	SEM	Mean	SEM	Mean	SEM
0.0000	100.00	0.00	100.00	0.00	99.97	0.01
0.9375	57.44	7.27	89.53	2.30	101.46	1.46
1.875	61.89	1.88	89.02	3.58	102.56	2.59
3.75	60.90	1.81	87.91	5.18	89.70	7.54
7.5	23.21	8.73	77.00	3.42	19.92	12.23
15	5.26	4.88	36.92	16.02	4.04	2.46
30	4.40	1.46	6.31	4.96	0.39	1.15
60	1.27	1.73	-0.75	1.57	-0.14	1.57

The mean ± SEM is shown (n≥3)

Table 2 The survival of Vero, WRL-68 and BALB/3T3 clone A31 cell lines after 24 hours oftreatment with Taxol

Taxol (µg/ml)	Cell type (% viability)					
	Vero		WRL-68		BALB/3T3 clone A31	
	Mean	SEM	Mean	SEM	Mean	SEM
0.00000	100.00	0.00	99.99	0.01	99.98	0.01
0.00001	71.70	7.78	93.82	3.80	97.13	3.52
0.0001	71.88	9.73	88.94	6.34	100.81	4.94
0.001	74.54	10.48	97.87	5.47	92.44	3.62
0.01	68.84	9.76	84.60	5.35	94.03	4.25
0.1	55.13	11.08	57.07	7.92	56.72	3.39
1.0	45.58	6.19	43.92	6.05	13.26	3.24

The mean ± SEM is shown (n≥3). Taxol is a cytotoxic drug that was used as a positive control

tem) statistical package (Springer-Verlag, USA).

The IC_{50} values for Nutri-pepper enhancer after 24 hours for the Vero, WRL-68 and BALB/3T3 clone A31 cell lines were 7.67 µg/ml, 14.39 µg/ml and 5.53 µg/ml, respectively, while these values for the positive control were 0.1179 µg/ml, 0.0238 µg/ml and 0.1048 µg/ml for the Vero, WRL-68 and BALB/3T3 clone A31 cell lines, respectively (**Table 3**). Since the IC_{50} values of Nutri-pepper enhancer towards all tested cell lines were much higher than those for Taxol, this indicates that this product is less toxic to kidney, hepatic and fibroblast cells.

Table 3 Mean half maximal inhibitory concentration (IC_{50}) values for test and control samples

Sample	IC_{50} (µg/ml)		
	Vero	WRL-68	BALB/3T3 clone A31
Nutri-pepper enhancer	7.67 ± 1.22	14.39 ± 2.78	5.53 ± 0.81
Taxol	0.1179 ± 0.0593	0.0238 ± 0.0122	0.1048 ± 0.0063

The MTT assay was used to determine the IC50 values for Nutri-pepper enhancer and Taxol with respect to their effects on cell viability. The mean ± SEM is shown

Discussion

Mouse fibroblasts (BALB/3T3 clone A31), and human kidney-like (Vero) and hepatic (WRL-68) cells were selected as representative cell lines because they play an important role in detoxifying the bulk of nutrients received [7]. Once received, metabolized nutrients are then released back into the bloodstream, and blood is pumped throughout the body from the heart. Therefore, human heart-like cells, WRL-68, were selected as being representative of primary human heart cells. Certainly, it would be possible to more reliably evaluate toxicity to humans by using various human-derived primary cells [8].

In the field of biomaterials, it is necessary to consider safety aspects, such as the elimination of cytotoxicity and other harmful effects of the material to be used [9]. By definition, the cytotoxicity of an agent means the toxicological risks caused by a material or its extract in a cell culture [10]. The interactions of the materials and their components with the cells at a molecular level are responsible for tissue reactions, such as inflammation, necrosis [11], immunological alterations, genotoxicity [12] and apoptosis [13].

In recent years, interest in *in vitro* systems as an alternative to animal experiments in toxicological research has been steadily increasing. Cytotoxicity testing includes numerous methods, both qualitative and quantitative. In this study, we used an indirect test, in which the metabolic activity (as assessed by the application of MTT) indicated the degree of Nutri-pepper enhancer-associated cytotoxicity. **Table 1** shows the effect of Nutri-pepper enhancer on the viability of fibroblast, kidney and hepatic cells as measured by the MTT test. MTT is a yellow water-soluble tetrazolium dye which is reduced by live cells to a purple formazan product that is insoluble in aqueous solutions. The amount of formazan generated is directly proportional to the number of viable cells. As can be seen from **Table 1**, exposure to 7.5 µg/ml Nutri-pepper enhancer over 24 hours of incubation resulted in a cytoviability of all tested cell lines of 77.00–19.92%.

The greatest inhibition effect was induced by 60 µg/ml and the weakest by 0.9375 µg/ml.

Although further statistical analysis showed no significant difference between the different concentrations, there was increased cytoviability with reduced extract concentration.

This means that exposure to a higher concentration tends to lower cell line cytoviability.

The IC_{50} is a measure of the effectiveness of a compound in inhibiting biological or biochemical functions. This quantitative measure indicates how much of a particular substance is needed to inhibit a given biological process by 50%. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC_{50}) [14, 15].

In this study, piperine, curcumin and leonurine, the target substances contained in the Nutri-pepper enhancer, were the main components contributing to the toxicity to the tested cell lines.

The results showed that this sample had a less toxic effect on fibroblasts, hepatic and kidney cells compared to the positive control, Taxol, indicating that this extract is not toxic to human health.

This finding was supported by the IC_{50} values of the sample and positive control against all the tested cell lines. The higher the IC_{50} value, the less toxic the agent. Since piperine, curcumin and leonurine can induce cell death in a concentration-dependent manner, as well as exhibit various pharmacological activities [16] such as antioxidation [17], anti-inflammation [18] and antitumour effects [19], our results showing cytotoxicity at a high concentration suggest that a therapeutic effect can be achieved without toxicity.

Conclusion

Biomaterials may have a potential risk to human safety. Therefore, accurate biological assessment of any proposed medical biomaterial is needed. Based on research into the toxicity of the pepper-based product Nutri-pepper enhancer on fibroblasts and kidney and hepatic cell lines, we conclude that Nutri-pepper enhancer at lower concentrations does not appear to be toxic to cells with its IC_{50} values after 24 hours ranging from 5.53–14.39 µg/ml, which are much higher than the positive control with IC_{50} values ranging from 0.0238–0.1179 µg/ml. From this finding, further research is proposed; specifically, to conduct an in vivo study to determine a comprehensive safety profile for the tested substance.

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