ANALYSIS OF ANTI-CANCER ACTIVITIES IN VITRO OF NUTRI-PEPPER ENHANCER

Yap Chin Ann, Sang Jam, Lawrance Tuah

Research and Development Division, Malaysian Pepper Board, Lot 1115 Jalan Utama, Pending Industrial Area, 93916 Kuching, Sarawak , Malaysia

IJASR 2021
VOLUME 4
ISSUE 1 JANUARY – FEBRUARY **ISSN: 2581-7876**

Abstract: Nutri-pepper enhancer contain active ingredient with anti-cancer properties with the IC_{50} value of 9.54-10.11μg/mL against ovary cancer cell line, 9. 44-9.76 μg/mL against breast cancer cell lines, 9.84 μg/mL against colorectal cancer cell line and 10.10 μg/mL against cervical cancer cell line. Morphological alterations in the cell lines after treatment were observed. This finding indicated that Nutri-pepper enhancer possessed effective cytotoxic activities against all the cancer cell lines. This research finding is in agreement with the criteria established by the American Cancer institute where the crude extract can be considered as a promising anticancer agent for further development into potential anticancer natural compounds based on the IC_{50} value lower than 30 μg/mL.

Keywords: (a) Nutri-pepper enhancer (b) Anti cancer (c) Ovary cancer (d) Breast cancer (e) colorectal cancer (d) cervical cancer

1.0 BACKGROUND

Several plant-derived compounds containing various type of plant phenolic, alkaloid, carotenoid etc. continue to be widely used in cancer therapy. Despite the continuing need for effective anti-cancer agents and the association of fruit and vegetable consumption with reduced cancer risk, edible plants are increasing considered as sources of anti-cancer drug. Although several plants have provided useful active ingredients for treatment of a variety of cancers, the anti-cancer potential of the plant kingdom remain largely unexplored. Of the approximately 400,000 land plant species known worldwide (Lughadha *et al*., 2016; Ulloa *et al*., 2017), it has been estimated that less than 20% have ever been studied for potential therapeutic effects, and a smaller percentage for anti-cancer activity (Cragg, 2009). These data suggest that an unknown number of plants with potentially useful anti-cancer compounds are waiting to be discovered.

Based on the usefulness and importance in among all the spices, black pepper is commonly referred as "The King of Spices". It is valued for its flavor, aroma, nutritional and medicinal uses making it an important commodity (Meghwal and Goswami, 2012). By its nature, it is spicy, aromatic and carminative. It is a natural antioxidant. It acts as anti-inflammatory, anticancer, antiperiodic and antipyretic (Ahluwalia and Raghav, 1997). Although many researches has reported the usefulness of pepper as a nutritional and medicinal foods, but the development of nutraceuticals were meagre. Moreover, with an increase of awareness among end-used on the potential of functional and nutraceuticals to improve health with fewer side effects and less expense than commonly use pharmaceuticals, nutraceutical industry become million dollar industry worldwide that worse to invest. Sarawak contains many crops with high medicinal values. The incorporation of pepper extract with some other active-ingredient derived from indigenous crop could further improve human health with multiple functions. This value added products have expected to catch the national and international markets if it is properly focused. A series of preclinical and non-clinical trial was conducted to determine the reliable evidence of the efficiency of nutraceuticals against a series of cancer cell lines. This project aims at investigating the effectiveness of Nutri-pepper enhancer (combination of pepper crop and other indigenous crop) as potentially useful anti-cancer nutraceutical product. This considered as one of government effort to stabilize the domestic pepper price through product diversification.

2.0 Objective

The objective of this study was to evaluate the anti-cancer activity of Nutri-Pepper Enhancer using a panel of different cancer cell line (colorectal, breast, ovarian and cervical) in vitro SRB assay.

 Copyright © 2021 IJASR All rights reserved (cc) BY

3.0 TEST SYSTEM

The SRB assay has been used since its development in 1990 (Skehan *et al.*, 1990) to inexpensively conduct various screening assays to investigate cytotoxicity in cell based studies (Vichai and Kirtikara, 2006). This method relies on the property of SRB, which binds stoichiometrically to proteins under mild acidic conditions and then can be extracted using basic conditions; thus, the amount of bound dye can be used as a proxy for cell mass, which can then be extrapolated to measure cell proliferation.

The protocol can be divided into four main steps: preparation of treatment, incubation of cells with treatment of choice, cell fixation and SRB staining, and absorbance measurement. This assay is limited to manual or semiautomatic screening, and can be used in an efficient and sensitive manner to test chemotherapeutic drugs or small molecules in adherent cells. It also has applications in evaluating the effects of gene expression modulation (knockdown, gene expression upregulation), as well as to study the effects of miRNA replacement on cell proliferation (Kasinski *et al.*, 2015).

4.0 MATERIALS AND METHODS

4.1. Sample

Nutri-Pepper enhancer was obtained from the Malaysian Pepper Board containing 3 major component: (turmeric extract (curcumin), black pepper extract (piperine) and *Leonurus japonicas* extract (leonurine)

4.2. Cancer cell lines

Human cervical cancer (HeLa ATCC® CCL-2™), human breast cancer (MCF-7 ATCC® HTB-22™ and T47D ATCC® HTB-133 ™), human colon cancer (HT-29 ATCC® HTB-38™) and human ovary cancer (SKOV-3 ATCC® HTB-77™ and A2780 ECACC catalogue no.93112519) cell lines were obtained from Aseacyte Sdn Bhd, Malaysia. HeLa, MCF-7 and T47D were cultured in Eagle's minimum essential medium supplemented with 1% L-glutamine, 10% foetal bovine serum, 1% penicillin streptomycin, 1% sodium pyruvate, and 1% non-essential amino acids. HT-29, SKOV-3 and A2780 were cultured in Eagle's minimum essential medium supplemented with 1% L-glutamine, 10% foetal bovine serum, 1% penicillin streptomycin, and 1% sodium pyruvate. All of the cell lines were stored in a humidified 5% CO_2 incubator.

4.3. Methods

4.3.1. Nutri pepper-enhancer extracts preparation

A 500-mL Scott bottle was prepared and filled with 80% ethanol to a total volume of 500 mL. A total of 50 g of ground Nutri pepper-enhancer was added to the solvent, followed by ultrasonic extraction (Ultrasonic Homogeniser Labsonic P, 400 W, Sartorius, AG) for 30 min, with a 5-minute pulse duration period and a 5-minute pulse interval period. The extraction was repeated for 3 cycles. The resulting Nutri pepper-enhancer extract was centrifuged at 3500 rpm for 10 min. The supernatant of the Nutri pepper-enhancer extract was collected and filtered; the pellet was discarded. The filtered supernatant was subjected to rotary evaporation (Rotavapor R-200, Buchi, Switzerland).

4.3.2. Cell preparation for assays

Cells were plated in 96-well plates ($5x10^4$ cells/well) for seeding. After 24 h of cell seeding, a partial monolayer was formed and the optimum cell confluence of 70% was achieved. The 96-well microtiter plates were treated with different concentrations of Nutri pepper-enhancer extract. About 0.1 mL of well mixed Nutri pepper-enhancer extract solution (31.25 µg/mL to 2 000 µg/mL) was added into the 96-well plates. Therefore, the final Nutri pepper-enhancer extract concentration was 2-fold diluted which range from 1.0 µg/mL to 625 µg/mL. Negative control was set as treated the cells with 0.9% of saline. Minimum essential medium was included as the blank control. All of the medium concentration was prepared in duplicate. The plates were incubated at 37 °C for 72 h before the cytotoxicity test is carried out.

4.3.3. SRB's colorimetric assay

Copyright © 2021 IJASR All rights reserved

An SRB assay was performed according to a slight modification of the procedure reported by Houghton and his co-workers [13]. Following the 72 h treatment, cytotoxicity was evaluated using the SRB assay. Twenty five microlitres of 50% trichloroacetic acid was gently added to the wells so that it formed a thin layer over the test compounds to reach an overall concentration of 10%. The plates were incubated at 4 °C for 1 h. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum and were then air dried. The air-dried plates were stained with 100 µL SRB and kept for 30 min at room temperature. The unbound dye was rapidly removed by washing four times with 1% acetic acid. The plates were then air-dried; 100 µL of 10 mmol/L Tris base was added to the wells to solubilize the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using an -linked immune sorbent assay plate reader (Biotek, United State) at a wavelength of 515 nm. The percentage growth inhibition was calculated using the following formula:

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

Where, At = Absorbance value of the test compound; Ab=Absorbance value of blank; Ac=Absorbance value of control.

The percentage of cell inhibition against the test compound concentration was plotted. The half maximal inhibition concentration (IC_{50}) was calculated based on the equation in the plotted graph.

4.4. Statistical analysis

All experiments were performed in duplicate and measurements were replicated two times (n=3). An analysis of variance was performed, and the average values were compared with Fisher's Multiple Comparison Test. Differences were considered statistically significant at $P<0.05$. All statistical analyses were performed using Minitab 16 for Windows.

5.0 RESULTS

The anticancer activity of Nutri-pepper enhancer was assessed using six (6) different cell lines, *HeLa*, MCF-7, HT-29 and SKOV-3 and A278, by using the SRB assay. The result showed that as the concentration of the sample increases, there is an increase in the cell growth inhibition with the maximum inhibition was reached at the concentration of 125 µg/ml for all the tested cancer cell lines. Thereafter, the efficacy was decreased at the concentration of 625 µg/ml (Table 1). The cytotoxicity or dose response rate of application experiment was useful in determining the optimum rate of Nutri-pepper enhancer for cancer cell lines inhibition control. The lower concentration (1 µg/ml) had shown higher percentages of cells viability, more cell lines survive than the higher concentration of 5, 25 and 125 µg/ml. The response of Nutri-pepper enhancer to rate applied is illustrated graphically. From the graph (Figure 1), it was clearly indicated that the percentage of cell viability decline drastically from 1 µg/ml to 125 µg/ml. The graph also showed that the optimum rate was arrived at 125 µg/ml by considering the flattening of percentage of cell viability curve between rates (Figure 1).

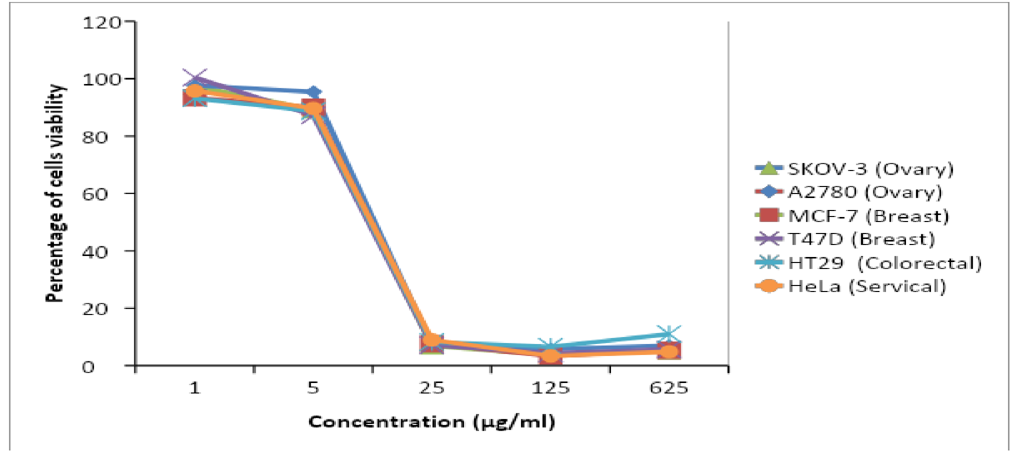

Figure 1: Dose response curves of the percentage of cells viability vs sample concentration in SKOV-3. A2780. MCF-7, T47D, HT-29 and HeLa cancer cell lines

Copyright © 2021 IJASR All rights reserved (cc) BY

IC_{50} is the acronym for "half maximal inhibitory concentration". IC_{50} value indicates the concentration needed to inhibit a biological or biochemical function by half (e.g. inhibition of enzymes, affinity to cell receptors). Amongst others, determination of IC_{50} is commonly calculated via linear interpolation. Based on the IC_{50} determination, it was found out that the IC_{50} for each of the tested cancer cell lines ranged between 9.54- 10.11 µg/ml (Table 1).

Modifications in the morphology of *HeLa*, MCF-7, HT-29 and SKOV-3 and A2780 after treatment at 72 h were observed (Figure 2). When all four cancer cells were exposed to cytotoxic components, two distinct modes of cell death were recognized, namely, apoptosis or necrosis. The majority of cells treated with Nutri-pepper enhancer showed features of apoptosis such as cellular shrinkage, membrane blebbing and apoptotic body formation as viewed under an inverted light microscope. Figure 2 show that Nutri- pepper enhancer caused the shrinkage and blebbing of cell membranes from *HeLa* and HT-29 after 72 h of treatment. On the other hand, most of the MCF-7 and SKOV-3 and A2780 membranes blebbed during shrinkage, and the apoptotic bodies were formed around cells after treating them with Nutri- pepper enhancer.

Table 1: percentage of cells viability of the L1 treated on SKOV-3, A2780, MCF-7, T47D, HT29 and HeLa cancer cell lines.

Cancer cell line	Sample concentration (µg/ml)	% of cells viability	S.D	IC_{50} (µg/ml), (n=3)
SKOV-3 (Ovary)	1	97.35	3.34	9.54±0.30
	5	89.23	2.96	
	25	6.94	0.56	
	125	3.77	0.05	
	625	5.14	0.17	
A2780 (Ovary)	1	97.64	0.66	10.11±0.054
	5	95.42	0.93	
	25	7.54	0.19	
	125	5.8	0.41	
	625	6.97	0.49	
MCF-7 (Breast)	1	93.34	1.52	9.76±0.36
	5	90.00	3.61	
	25	7.66	0.46	
	125	3.37	0.03	
	625	5.31	0.23	
T47D (Breast)	1	100.40	2.18	9.44±0.16
	5	87.23	1.02	
	25	7.13	0.66	
	125	4.32	0.26	
	625	5.68	0.31	
HT29 (Colorectal)	1	93.18	0.98	9.84±0.081
	5	88.71	1.50	
	25	8.23	0.21	
	125	6.58	0.17	
	625	11.03	0.03	
HeLa (Servical)	1	95.75	1.58	10.10±0.31
	5	89.65	2.20	
	25	8.98	0.61	
	125	3.41	0.11	
	625	4.85	0.30	

Copyright © 2021 IJASR All rights reserved (cc) BY

Treatment	Ovary cancer, SKOV-3	Breast cancer, MCF-7	Colorectal cancer, HT29	Cervical cancer, HeLa
Before				
After treatment				

Figure 2: Morphological changes of cervical cancer (HeLa), breast cancer (MCF-7), colorectal cancer (HT-29) and ovary cancer (SKOV-3) treated with the Nutri pepper-enhancer for 72 h.

CS: Cellular shrinkage; BL: Membrane blebbing; IC: Intact cell structure; DC; Dissociated from cell structure (Magnification for Hela 40x, magnification for MCF-7, HT-29 and SKOV-3 10x).

6.0 DISCUSSION

Plants have a nearly unlimited capacity to generate compounds that fascinate researchers in the quest for new and novel chemotherapeutics (Rubal *et al.*, 2010). The persistent search for new anti-cancer compounds in plant medicines and traditional food is a realistic and promising strategy for its prevention (Andersen *et al.*, 2015). Therefore, the newly developed pepper based nutraceutical, namely Nutri Pepper enhancer was evaluated by using SRB assays. Mahalingam *et al.*, 2016 indicated that under similar experimental conditions and within the limits of the applied data analyses, the SRB has been widely used to investigate cytotoxicity in cell based studies and it is the method of choice for high cost-effective screenings. Since this method does not rely on measuring metabolic activity [*e.g.*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT], the steps required to optimize the protocol for a specific cell line are substantially simplified. Furthermore, both MTT and SRB assays generally yielded similar results. Manosroi and his co-workers suggested that sample that had IC_{50} value of less than 30 µg/mL could be a possible candidate for further development to cancer therapeutic agent and a test component

Copyright © 2021 IJASR All rights reserved (cc) BY

with IC_{50} value between 9.54 and 10.11 μg/mL was considered to have highly potential to be developed into a cancer therapeutic agent (Itharat *et al.*, 2004). Thus, Nutri pepper enhancer could be a potential source for the treatment components of cancer.

To date, there was no reported research on cytotoxic properties of pepper based products. From the research finding, it was found out that the nutri-pepper enhancer possess anti-cancer activities against ovary, breast, colorectal and cervical cancer which is might probably due to the present of plant phenolic and alkaloid compounds. According to preliminary study conducted, there were three main phenolic compounds identified in the nutri-pepper enhancer, namely, piperine, cucumin, and leonurines. The efficacy of this product as anti-cancer drug was further support by many studies conducted on cell cultures and animal models indicated that polyphenols are the main phytochemicals with antioxidant and anti-proliferative properties from higher plants (Huang et al., 2012). These molecules may act as cancer-blocking agents, preventing the initiation of the carcinogenic process as cancer-suppressing agents and inhibiting cancer promotion and progression (Berrington and Lall, 2012). This finding showed that phenolic compounds in Nutri- pepper enhancer are responsible for cytotoxic properties.

7.0 CONCLUSION

This study provided evidence showing that Nutri-pepper enhancer possessed effective cytotoxic activities against HeLa, MCF-7, T47D, SKOV-3, A2780 and HT-29. These properties were likely to be related to the phenolic and alkaloid constituents. From the present studied it had been concluded that Nutri-pepper enhancer enable exhibiting the potential cytotoxic action on all tested cell line which was proved by using standard SRB assay and it was found that the highest cell growth inhibition (more than 95%) was at 125 μg/ml with the IC_{50} value ranged between 9.44-10.11μg/ml. The criteria of American Cancer Institute considered a crude extract promising for further development of a potential anticancer natural compound with a higher cytotoxic activity based on the IC_{50} values lower than 30 μg/mL (Itharat *et al.*, 2004). Based on these criteria, this product showed a potential that may be developed as a new anticancer drug. Therefore, this study suggested that Nutri-pepper enhancer may be used as inexpensive and easily accessible sources of potential natural anti-cancer agents. Further investigations on a larger number of cancer cell lines and *in vivo* studies should be conducted to further confirm the effectiveness of this product as anti-cancer nutraceutical products.

8.0 References

1. Ahluwalia, V.K., Raghav, S. (1997). Comprihensive experimental chemistry. New Age International Publishers, New Delhi.6. Vijayan KK, Thampuran RVA (2000) Pharmacology, toxicology and clinical applications of black pepper. Harwood Academic Publishers.
2. Andersen, T.B., Lopez, C.Q., Manczak, T., Martinez, K., Simonsen, H.T. (2015). Thapsigargin—from Thapsia L. to mipsagargin. Molecules, 20(4): 6113-6127.
3. Bader, A. G. and Slack, F. J. (2015). A combinatorial microRNA therapeutics approach to suppressing non-small cell lung cancer. Oncogene 34(27): 3547-3555.
4. Berrington, D., Lall, N. (2012). Anticancer activity of certain herbs and spices on the cervical epithelial carcinoma (HeLa) cell line, Evidence-Based Complementary and Alternative Medicine, 20(12), 11-22.
5. Cragg, G.M., Grothaus, P.G., Newman, D.J. (2009). Impact of natural products on developing new anticancer agents. Chem. Rev., 109(7): 3012-3043.
6. Huang, M., Lu, J., Huang, M., Bao, J., Chen, X., and Wang, Y. (2012). Terpenoids: natural products for cancer therapy, Expert Opinion on Investigational Drugs, 21(12), 1801–1818
7. Itharat, A., Houghton, P. J., E., Eno-Amooquaye, P. J., Burke, J. H., Sampson, C.and Raman A. (2004). *In vitro* cytotoxic activity of Thai medicinal plants used traditionally to treat cancer, *Journal of Ethnopharmacology*, 90(1), 33–38.
8. Lughadha, E.N., Govaerts, R., Belyaeva, I., Black, N., Lindon, H., Allkin, R., Magill, R.E., Nicolson, N. (2016). Counting counts: Revised estimates of numbers of accepted species of flowering plants, seed plants, vascular plants and land plants with a review of other recent estimates. Phytotaxa, 272(1): 82-88.
9. Mahalingam, D., Wilding, G., Denmeade, S., Sarantopoulas, J., Cosgrove, D., Cetnar, J., Bruce, J., Kurman, M., Allgood, V.E., Carducci, M., (2016). Mipsagargin, a novel thapsigargin-based PSMA-activated prodrug: results of a first-in-man phase I clinical trial in patients with refractory, advanced or metastatic solid tumours. Br. J Cancer, 114(9): 986-994.

 Copyright © 2021 IJASR All rights reserved (cc) BY

10. Meghwal, M., Goswami, T.K. (2012) Nutritional Constituent of Black Pepper as Medicinal Molecules: A Review. 1(129) 129-135.
11. Rubal, J.J., Moreno-Dorado, F.J., Guerra, F.M., Jorge, Z.D., Galan, M.C., Salido, G.M., Christensen, S.B., Sohoel, H., Massanet, G.M. (2010). A phenylpropanoid, a slovenolide, two sulphur-containing germacranes and Ca^{2+}-ATP_{ase} inhibitors from Thapsia villosa. Planta Med., 76(3):284-290.
12. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T.,Bokesch, H., Kenney, S. and Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 82(13): 1107-1112.
13. Ulloa, U.C., Acevedo-Rodriguez, P., Beck, S., Belgrano, M.J., Bernal, R., Berry, P.E., Brako, L., Celis, M., Davidse, G., Forzza, R.C., Gradstein, S.R., Hokche, O., Leon, B.(2017). An integrated assessment of the vascular plant species of the Americas. Science. 2017; 358(637): 1614-1617

Copyright © 2021 IJASR All rights reserved