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SOME APPLICATIONS OF CELL-BASED PHARMACOLOGICAL SCREENING FOR MEDICINAL PLANTS IN VIETNAM

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ABSTRACT

Background: Vietnam has a wide variety of plants traditionally used as treatment for mild to severe diseases. In order to add up scientific evidences for utilization of medicinal plants in disease prevention and treatment, cell based-assay has gradually been applied to speed up drug discovery process in terms of screening a large amount of sample in a short time with reliable results, while investigating drug's action mechanism at molecular and cellular levels. Objectives: This article reviewed the existing cell-based assays that could be applied to screen anticancer, hepatoprotective and renoprotective effects of medicinal plants in Vietnam. Methods: Bioassays used to screen for anticancer activity consist of anti-proliferation test, apoptosis investigation, inflammation and oxidative stress evaluation of in cancer cells. Hepatoprotective and renoprotective effects are evaluated through several assays including cell proliferation, oxidative stress, necrosis, apoptosis activation and Western blotting. Results: In Vietnam, Annona glabra, Ehretia longifolia, Uvaria cordata, Stephania glabra, Morus alba, Paramignya trimera… have been studied for their anticancer effect. Some medicinal plants that have hepatoprotective effects are Eclipta alba, Phyllanthus amarus, Ocimum sanctum whole plant, Andrographis peniculata, Houtyna cordata ariel part, Curcuma longa rhizome, Taxaracum officinale, Glycyrrhiza glabra root, Moringa oleifera, Camelia sinensis, Cynara scolymus leaves… However, there are few researches on screening the renoprotective effect of medicinal plants. Conclusions: Because the rate of hepatic or renal diseases and cancer has increased in recent years in Vietnam, cell-based screening should be applied in order to best utilize the rich sources of medicinal plants.

Keywords: anticancer, cell-based screening, hepatoprotective, medicinal plant, renoprotective.

I. INTRODUCTION

Plants' curative effects have been either recorded in books or simply passed from generations to generations by word of mouth, gradually forming people's usage habit. According to World Health Organization, 80% of the people living in rural areas depend on medicinal herbs as primary healthcare system [17]. These plant products are in continuous demand and have an edge over other products in the area of drug discovery and therapeutics due to their high efficacy, safety and easy availability [11].

An early part of the experimental process often involves screening a large number of compounds using defined biochemical assays. However, the effect of a drug on an organism is complex and involves interactions at multiple levels that cannot be predicted using biochemical assays. Trying to understand this complexity has contributed to an increased use of cell-based screening assays as more biologically relevant surrogates to predict the response of the organism. In addition, at some point in the drug discovery process, predicting cellular toxicity is important. Eukaryotic cell culture is accepted as the model system of choice to get a first approximation of toxicity. Furthermore, advances in assay chemistries and signal detection technology have allowed miniaturization of cell-based assays, making it more convenient to perform dose-response experiments during primary screens [15].

Located in a tropical region, Vietnam is granted with rich sources of medicinal plants. Although there are reports from many researchers on the anticancer, hepatoprotective or renoprotective agents in various types of plant, these scientific data are inadequate. This article reviewed some applications of cell-based pharmacological screening in order to build a firm scientific evidence for Vietnamese medicinal plants.

II. ANTICANCER EFFECT

Cancer is one of the most severe health problems worldwide. Deaths due to cancer are projected to continuously increase and it has been estimated that there will be 11.5 million deaths in the year 2030 and 27 million new cancer cases and 17.5 million cancer deaths are projected to occur in the world by 2050. Cancer cell is abnormal and able to divide without control and to invade other tissues. Carcinogenesis is a multi-step process consisting of tumor initiation, promotion and progression, resulting in DNA damage, oxidative stress, and chronic inflammation. Conventional chemotherapy treatment of cancer includes mainly alkylating agents, antimetabolites, antitumor antibiotics, platinum analogs and natural anticancer agents. However, due to the adverse or toxic side effects of cancer chemotherapy, discovery of new anticancer agents derived from nature, especially plants, is currently under investigation [6].

Plant secondary metabolites such as isoprenoids, phenolics and alkaloids, have been demonstrated to be the leading providers of novel anticancer agents [6]. In Vietnam, *Annona glabra, Ehretia longifolia, Uvaria cordata, Stephania glabra, Morus alba, Paramignya trimera…* have been studied for their anticancer effect.

In vitro bioassays used to screen for potential anticancer activity consist of cell culture systems in which neoplastic cell lines (Table 1) have been established from human or other animal tumors. Ability of the tested compounds to inhibit the proliferation of these cancer cells in culture is an indication of potential value. Other parameters of the assays are apoptosis investigation, inflammation and oxidative stress evaluation [2,13, 14].

Table 1: Common cancer cell lines in cell-based screening

Types of cell	Name
Human breast cancer cell	MCF-7, MDA-MB
Human lung cancer cell	A549, NCI-417, Lu
Human hepatocarcinoma	HepG2
Human cervical epithelioid cells	Hela
Human muscle rhabdomyosarcoma	RD
Human prostate cancer cells	IO 26, LNCaP, 22Rv1, PC-3 and DU145

III. RENOPROTECTIVE EFFECT

The kidney maintains precise body and/or extracellular electrolyte, fluid balance and blood pressure homeostasis [22]. Due to its physiological functions, kidney is exposed to various xenobiotic compounds with a condensed concentration resulting in the damage in the renal tissue, acute kidney injury and chronic kidney disease. The pathology pathways can be explained by the interaction between the intracellular macromolecular and the toxic xenobiotic and by the formation of reactive oxygen species (ROS), which causes cell stress, lipid peroxidation and cellular damage [7].

Medicinal plants can exert renoprotective effect due to its dose dependent antioxidant effect. This effect is mediated by scavenging of free radicals, decreasing formation of ROS and inhibition of fatty acid peroxidation. It also involves anti-inflammatory and antiapoptotic actions through interference with nuclear factor kappa-B (NF-κB), modulation of inducible nitric oxide and decreases in cyclooxygenase-2 expression [20] In Vietnam, there are few researches on screening the renoprotective effect of medicinal plants.

LLC-PK1, NRK, and HK-2 are used *in vitro* to screen renoprotective effect of sample. Cells are treated with plant sample alone or combined with renotoxicity agent (cisplatin, cyclosporine, paracetamol, gentamycin, metformin…). Renoprotective effect is evaluated through several assays including cell proliferation, oxidative stress, necrosis, apoptosis activation and Western blotting [15, 23].

IV. HEPATOPROTECTIVE EFFECT

The liver is one of the most vital organs that metabolites nutrient; digests, stores and excretes of the products. Moreover, it is an integral part of drug metabolism and removal of xenobiotics from the body thus protecting it against foreign substances by detoxifying and eliminating them. As easily being exposed to various toxic agents like carbon tetrachloride, thioacetamide, certain antibiotics, alcohol, aflatoxin or reactive

oxygen species during their metabolism, hepatocytes can be severely damaged by inducing lipid peroxidation and other oxidative damages. Liver diseases are classified as acute or chronic hepatitis (inflammatory liver diseases), hepatosis (non- inflammatory diseases), cirrhosis (degenerative disorder resulting in fibrosis of liver) and cancer [11].

Medicinal plants, involving one or more mechanisms, help hepatocytes/liver in proper functioning by increasing in antioxidant level/decreasing in oxidants (ROS formation), inhibition of cytochrome P450s, increasing or decreasing level of liver enzymes, reduced peroxidation / lipid peroxidation (MDA), and increase in level of glutathione [9]. Some medicinal plants that have hepatoprotective effects in Vietnam are *Eclipta alba, Phyllanthus amarus, Ocimum sanctum* whole plant, *Andrographis peniculata, Houtyna cordata* ariel part, *Curcuma longa* rhizome*, Taxaracum officinale, Glycyrrhiza glabra* root, *Moringa oleifera, Camelia sinensis, Cynara scolymus* leaves…

HepG2 is a common cell line in cell-based screening for hepatoprotective effect. Cells are treated with plant sample alone or combined with hepatotoxicity agent (CCl_4, ethanol, fatty acids, and glucose). Hepatoprotective effect is evaluated through several assays including cell proliferation, intracellular lipid accumulation, oxidative stress, necrosis, and apoptosis activation [5, 9].

V. CELL-BASED ASSAYS

Cell culture

Cells are seeded and cultured in suitable medium in humidified atmosphere of 5% CO_2 at 37°C to attain confluency. Cells then are trypsinized, harvested and counted using trypan blue, and seeded in 6 or 96-well-plates at a fixed concentration.

Cell proliferation assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
Cell viability is evaluated as mitochondrial succinate dehydrogenase (SDH) activity, a marker of viable cells using MTT test. Briefly, SDH activity is detected after 3h incubation in culture medium without serum containing MTT, which is converted into formazan dissolved in acidified isopropanol. The produced purple solution is spectrophotometrically measured at 570 nm on microplate reader [4].

Sulforhodamine B (SRB) assay
The antiproliferative SRB assay estimates cell number by staining total cellular protein with the SRB dye. Cells are fixed by 10% tricloroacetic acid at 4°C for 1 hour. The supernatant is then discarded. The plates are washed five times with distilled water. SRB solution is added and incubated at room temperature for 30 minutes. The unbound SRB is removed quickly by washing the wells five times with 1% acetic acid and then air dried. 100 µL of Tris buffer (0.01 M, pH 10.4) is added and shaken gently for 5 minutes on a mechanical shaker. Optical density is recorded on ELISA reader at 515 nm [17].

Oxidative prevention

Gluthathion content (GSH)
GSH is an antioxidant tripeptide which can decrease in the presence of ROS. Briefly, cell lysates in cold KCl 1.15% are homogenized with Tris-HCl 25 mM pH 7.4

(2:1, v/v), then incubated in 60 min at 37 ^{o}C. Later, mixture is added TCA 10% (1:1, v/v) then is centrifuged. 100 µL the supernatant is mixed with 180 µL EDTA- phosphate buffer and 20 µL 5,5'-dithio-bis 2-nitrobenzoic acid 5 mM (Ellman reagent). GSH levels are spectrophotometrically measured at 412 nm and deduced from a calibration curve and corrected from protein level of cell lysates. The protein content is also determined using BSA as standard. Results are expressed as µM GSH/mg protein [16].

Lipid peroxidation – Malonyl dealdehyde (MDA)

Accumulated intracellular lipid peroxidation is measured as MDA equivalent generated, as an indicator of lipid peroxidation in cultured cell lysates, using Lipid peroxidation assay kit (Abcam). The MDA in the sample reacts with thiobarbituric acid (TBA) to generate a MDA-TBA adduct which can be easily quantified colorimetrically (OD = 532 nm) [12].

Intracellular lipid droplet accumulation

O red oil staining

To measure intracellular lipid droplet accumulation, cells are stained by the O red oil method. Cells are rinsed with PBS and fixed with formalin 10%, rinsed with water followed by 60% isopropanol, stained with O red oil solution. Excess stain is removed by washing with water. The stained cells are observed under microscopy. Later, isopropanol 100% is added to dissolve stained oil droplets. The dye-triglyceride complex is spectrophotometrically measured at 500 nm [18].

Anti-inflammatory evaluation

Determination of nitric oxide (NO) and TNF-α production

The concentration of NO in culture supernatant is measured in the form of nitrite, a major stable product of NO using Griess reagents assay. After incubation, 100 µL culture supernatant of collected and mixed with equal amount of Griess reagents. NO production is measured by absorbance at from 520 to 550 nm by using ELISA microplate reader. Level of TNF-α secretion in culture medium is measured using available human TNF-α assay kit (Biosource, Camarillo, CA). Total culture supernatant is diluted with working reagent (1:2); 100 µL of diluted sample is used for ELISA assay. The total secreted amount of TNF-α level is estimated by absorbance at 450 nm by using ELISA micro-plate reader [12].

Cell necrosis

Lactate dehydrogenase (LDH)

Extracellular LDH is considered as an index of cell necrosis. LDH activity is measured in culture medium, using "Cytotoxicity detection LDH" kit. LDH participates in a coupled reaction which converts a yellow tetrazolium salt into a red, formazan-class dye which is measured by absorbance at 492 nm. A LDH standard curve (0 - 1000 mU/mL) is used for quantification of enzyme activity [10].

Aspartate transaminase (AST) – Alanine transaminase (ALT)

ALT and AST levels in culture medium are measured by available assay kits (Randox Laboratories, Antrim, UK). After incubation, culture supernatant is removed and

the total ALT and AST levels are measured using an ELISA microplate reader at 340 nm according to the supplier instructions. Results are expressed as U/L/mg protein [12].

Apoptosis investigation

Acridine orange/ethidium bromide (AO/EB) fluorescence staining

Changes in cell morphology are evaluated using AO/EB fluorescence staining. Briefly, cells are stained with dye mixture containing 100 µg/mL acridine orange and 100 µg/mL ethidium bromide in PBS, and then washed with PBS. After staining, cells are visualized immediately under a fluorescence microscope [1].

Determination of percentage (%) of DNA fragmentation

Briefly, DNA from the cells is released into the lysis buffer [0.2% Triton X-100, 10 mM Tris-HCl, and 10 mM EDTA (pH 8.0)] by rupturing the nucleus. The supernatant receive after centrifugation at 13000 rpm for 10 min at 4°C, contains fragmented DNA while the intact DNA is in the pellet. The amount of DNA in both the supernatant and pellet is estimated by diphenylamine (DPA) assay. The percentage of DNA fragmentation is calculated as the ratio of DNA in supernatant and DNA in pellet [3].

Detection of DNA fragmentation by agarose gel electrophoresis

Fragmented DNA is released into the lysis buffer (0.2% Triton X-100, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) by rupturing the nucleus. DNA in the supernatant is extracted using a mixture of phenol: chloroform: isoamyl alcohol (25:24:1-v/v/v). DNA is precipitated overnight with absolute ethanol and 300 mM NaCl, dissolved in TE buffer [10 mM Tris-HCl pH 8.0 and 1 mM EDTA containing RNase A (0.1 mg/mL)]. The samples are analyzed electrophoretically on 2% agarose gel containing 0.01% SYBR Green [1].

Flow cytometry (FCM) with propidium iodide (PI)

Cells are harvested by centrifugation and are fixed gently by addition of 70% ethanol at 4°C overnight and then re-suspended in phosphate-buffered saline (PBS) containing 40 µg/ml PI and 0.1 mg/ml RNase and 0.1% Triton® X-100 in a dark room. After 30 minutes at 37°C, the cells are analyzed with a flow cytometer equipped with an argon ion laser at 488 nm wavelength. Apoptosis is determined and analyzed. Annexin V-FITC and PI double staining kit from PharMingen (San Diego, CA, USA) are used for apoptotic cell quantification [8].

Protein extraction and Western blotting analysis

Cells are collected after treatment, washed with PBS three times, lysed in cell lysis buffer and then centrifuged at 13 000 × *g* for 10 min at 4°C. The extracted protein sample is added in the same volume of sample buffer and subjected to denaturation at 100°C for 10 min, then electrophoresed on 100 g/L or 60 g/L SDS-PAGE at 100 mA for 3 h, and finally transferred onto PVDF membrane. The PVDF membrane is treated with tris buffer saline tween (TBST) containing 50 g/L skimmed milk at room temperature for 2 h, followed by incubation with the suitable primary antibodies with appropriate dilution rate (p53, cytochrome c, PPARγ, NF-κB, Bcl-2, Bax…) at 37°C for 2 h or at 4°C overnight.

After being washed with TBST for 30 min, the corresponding secondary antibody is added and incubated at room temperature for 1 h. The membrane is then washed three times for 15 min each with TBST. Fluorescence is visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL). The results are analyzed with Image analyzer and the product of area and optical density is expressed as integral absorbance (IA) [21].

VI. CONCLUSIONS

The urge to produce a safe, low-cost and effective plant-derived medicine puts cell-based screening as an important initial step in drug development progress. It is an effective and reliable *in vitro* method to screen for a large amount of drug candidates in a short time. Besides investigating drug candidates' biological effects, cell-based screening also allows scientists to elucidate the mechanism of drug actions at molecular and cellular level. Because the rate of hepatic or renal diseases and cancer has increased in recent years in Vietnam, cell-based screening should be applied in order to best utilize the rich sources of medicinal plants.

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DETERMINATION OF VITAMIN C IN MULTIVITAMIN EFFERVESCENT TABLETS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT:

Background:*Multivitamin effervescent tablet is favorite supplement dietary and has been produced by many manufacturers. To verify the amount of vitamin C in multivitamin effervescent tablets is necessary to assure customer's health. But up till now, in present Vietnamese pharmacopoeia, there has not been quantitative method for effervescent tablet yet. **Objective:** we develop a high-performance liquid chromatography (HPLC) method for determination of vitamin C as main ingredient in multivitamin effervescent tablet with high sensitivity and short analytical*