Cytomic Screening of Immuno-Modulating Activity Compounds from *Calocedrus formosana*

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Abstract: Procedures for cytoxic screening were developed for identifying compounds with immuno-modulating properties from the crude extracts of natural products. Human peripheral blood mononuclear cells (hPB-MNCs) were first cultured with different natural crude extracts for 12 hours in culture media. By analyzing the expression of early activation CD69 marker, the potential immuno-activating properties of ethanol extracts of *Calocedrus formosana* were observed. By the double staining of antibodies recognizing CD69 and specific cell type markers, the increase of CD69 expressions was observed in CD3 and CD14 cell populations. To examine the immuno-activating properties in CD3 T cells and CD14 monocytes, the extracts were further purified. From NMR and mass spectra, sugiol was identified as a pure functional compound, and its immuno-enhancing activities were confirmed by CD69 expressions in the affected cell populations. Furthermore, to clarify the sugiol-affected subpopulations in-CD3 T cells, CD3 T cell activation in association with increase in CD8 cytotoxic T cells subpopulation was observed. To address the effect of sugiol on each isolated cell population, we found that the expression of CD69, CD80, and CD86 increased in CD14 monocytes upon exposure to sugiol, whereas for CD3 T cells, sugiol failed to induce the expressions of CD69 and CD25. However, T cell activation by co-culturing monocytes and T cells suggests that the sugiol activation of T cells in hPB-MNCs involved the accessory mechanisms of sugiol-primed monocytes. Therefore, cytoxic screening as a multiple-parameter screening strategy reveals the plasticity for immuno-functional studies, leading to the applications to discover new drugs of specific immuno-modulating activities.

Keywords: Cytoxic screening, adult human peripheral blood mononuclear cells (hPB-MNCs), *Calocedrus formosana*, sugiol, early activation marker CD69, CD8 cytotoxic T cells.

INTRODUCTION

Natural products have played an important role throughout the world in treating and preventing human diseases throughout the history in different parts of the world [1,2]. In particular, plants or herbs are common sources to discover novel bioactive molecules. Research on immuno-modulation by natural products is of key interest for anti-infective therapies and health cares. Natural extracts from grape seeds [3-5], green tea [6-8], *Triticum aestivum* [9,10] and *Ganoderma lucidum* [11-14] have been proven to contain diverse immuno-modulating activities either directly affecting the pathogen or in most cases indirectly stimulating or regulating the natural and adaptive defense mechanisms of the hosts [15]. *Calocedrus formosana* Florin (Cupressaceae), the targeted specimen of this study, is an endemic tree that grows an altitude of 800-1500 meters in the Central Mountains of Taiwan. Extracts from the bark of *C. formosana* have been

reported to possess some bio-active effects, such as anti-tumor [16], anti-oxidation [17], anti-inflammation [18], anti-termitic and anti-fungal activities [19]. However, the immuno-modulating properties of *C. formosana* extracts have not yet been elucidated.

Immune system is a complex network involving different types of cells in an organism. The immune cells undertake diverse approaches to communicate with one another [20, 21]. The direct cell interactions are established between cell surface ligands and receptors. By secreting various soluble mediators, such as cytokines, chemokines, and hormones, the indirect interfaces are performed [22]. For this reason, it is essential to choose an appropriate cell to give a comprehensive assessment for immuno-modulating activity investigation. In this study, we chose adult peripheral blood mononuclear cells (hPB-MNCs) for examining the immuno-modulating properties of *C. formosana* extracts. To select this type of cell includes two reasons. First, being a multiple cell type, hPB-MNC allows a systematic approach for screening. The major fractions of isolated hPB-MNCs contain both nonspecific and specific immune cells. The former are monocytes/macrophages and natural killer (NK) cells; and the later are T and B lymphocytes. Furthermore, various monoclonal antibodies for the cluster of differentiation (CD) markers are available for providing detailed description of

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phenotypic changes. The dynamic changes of CD markers, expressed on the cell surface of hPB-MNCs, reflect the physiological status of distinct cell populations [23]. Activated lymphocytes are capable of expressing a number of CD markers, such as CD25, CD69, CD71, CD80, CD86 and HLA-DR. These markers are minimally expressed or absent in other fractions of hPB-MNCs [24, 25].

In this study, we integrated combinatorial CD marker detections with systematic screening to yield cytomic screening for examining the immuno-related bioactivities of tested samples, since cytomics is the study of cell systems with special focus on the determination of molecular phenotypic description at the single cell level [26, 27]. The activation marker CD69 combined with four cell type-specific markers (CD3 for T cells, CD19 for B cells, CD14 for monocytes and CD56 for natural killer [NK] cells) were adopted as targets for evaluation. The combinatorial CD markers were recognized by different fluorochrome-conjugated monoclonal antibodies (mAb) and then detected by flow cytometric technology for high-throughput, a fluorescence-based multiparametric imaging examination [26]. By cytomic screening, sugiol was identified as the compound with immuno-activating properties from *C. formosana* extracts. The results of this study reveal that cytomic screening could serve as a platform for future library screening or chemical mining of components with immuno-modulating properties from crude natural extracts. This approach can lead to the discovery of potential drugs with immune-regulatory properties.

MATERIALS AND METHODS

Materials

The samples of *Calocedrus formosana* were collected in September 2003 from the Lien Hua-Chin Research Center located in Nantou County, Taiwan. The species was identified by Mr. Yen-Ray Hsui of Taiwan Forestry Research Institute, Taiwan. Voucher specimens (reg. #WCCL-04-B006) were deposited at the laboratory of wood chemistry in the School of Forestry and Resource Conservation, National Taiwan University, Taiwan. Normal human peripheral bloods (hPB-MNCs) were provided by Taipei Blood Center. RPMI-1640 medium powder, Ficoll-Hypaque, phorbol 12-myristate 13-acetate (PMA) and LPS (from *Escherichia coli* 0111:B4) were purchased from Sigma Co. The fluorochrome-conjugated monoclonal antibodies, including anti-CD3-FITC, anti-CD4-FITC, anti-CD8-FITC, anti-CD14-FITC, anti-CD19-FITC and anti-CD56-FITC, were purchased from Serotec. Anti-CD25-PE, anti-CD69-PE, anti-CD80-PE and anti-CD86-PE were obtained from BD Biosciences. Human primary monocytes and T cells isolation kits (Monocyte Isolation Kit II, human [130-091-153] and Pan T Cell Isolation Kit II, human [130-091-156]) were purchased from Miltenyi Biotec. The other chemicals and solvents used in this study were of the highest quality available.

Methods

Extraction and Purification of *Calocedrus formosana* and Identification of Sugiol

The bark particles of *C. formosana* (10 kg d. w.) were soaked in 95% v/v ethanol (10 days, repeated 3 times) at room temperature. The specimens were then concentrated to give the alcoholic extracts (AE; ca. 410 g). AE (205 g) was

successively extracted with *n*-hexane, ethyl acetate (EtOAc), dichloromethane, butanol, and deionized water. The yield of *n*-hexane-soluble fraction was about 64.8 g. The *n*-hexane fraction was purified by silica gel chromatography (*n*-hexane/EtOAc: 95/5, 90/10, 85/15, 80/20, 75/25, 70/30, 60/40, 40/60, 20/80, 0/100, followed by elution with EtOAc/ethanol: 100/0, 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 20/80, 0/100) to give 22 subfractions. Each eluted volume of subfractions was 1000 mL, except for H1 (*n*-hexane subfraction 1) which was 4000 mL. The collected subfractions were dried by rotavapor, leading to a white solid H5 subfraction (678 mg). 50 mg Sugiol was purified from H5 by recrystallization and semi-preparative HPLC (Hitachi L-7100 pump, a UV detector model Jasco UV 975, $\lambda = 254$ nm) in a Zorbax SIL column (25 cm in length, 4.6 mm i.d., 5.0 μ m). The separation conditions were as follows: flow rate, 2 mL/min; mobile phase, EtOAc/EtOH = 1/1. The structure of sugiol was determined by electrospray-MS and $^1\text{H-NMR}$. The mass spectra (MS) were obtained on a Finnigan MAT-95S mass spectrometer. electrospray-MS: m/z calcd. for $\text{C}_{20}\text{H}_{28}\text{O}_2$: 300; found: 300. The optical rotation was determined using a Horiva STA-300 polarimeter $[\alpha]_{\text{D}}^{25}$: $+28.3^\circ$ (c 1.0, CHCl_3). The NMR spectra were recorded on a Bruker Avance-300 MHz FT-NMR. $^1\text{H-NMR}$ (CDCl_3): $\delta = 7.89$ (s, 1H, H-14), 6.68 (s, 1H, H-11), 3.13 (sept, 1H, $J = 7.0$ Hz, H-15), 2.62 (dd, 1H, $J = 4.5$, 1.8 Hz, H-6 α), 2.18 (dd, 1H, $J = 13.0$, 18.0 Hz, H-6 β), 1.83 (dd, 1H, $J = 4.5$, 13.0 Hz, H-5), 1.24 (d, 3H, $J = 7.0$ Hz, H-17), 1.20 (d, 3H, $J = 7.0$ Hz, H-16), 1.19 (s, 3H, H-20), 1.00 (s, 3H, H-19), 0.99 (s, 3H, H-18). These values were confirmed with previously reported values.

Isolation and Stimulation of Adult Human Peripheral Blood Mononuclear Cells (hPB-MNCs)

Adult human peripheral blood mononuclear cells (hPB-MNCs) were separated from buffy-coats obtained from healthy donors by Ficoll-hypaque centrifugation. The mononuclear cells were washed by 2 mM EDTA containing PBS, and adjusted to the cell density of 2×10^6 cells/mL in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 100 unit/mL penicillin, and 100 unit/mL streptomycin as the complete medium (CM). Cells were incubated in normal culture condition at 37°C and 5% CO_2 for 24 h to equilibrate followed by drug stimulation. 1×10^6 hPB-MNCs cultured in 500 μ L CM were treated with 100 μ g/mL samples collected from the processes of purification from the crude extracts of *C. formosana* and 100 μ M identified sugiol and incubated under normal culture conditions. PMA and LPS as positive response controls were treated at 20 ng/mL and 1 μ g/mL respectively. An equal volume of vehicle solvent (extractions dissolved in DMSO) was added to the control culture. After 12 h of stimulation, cells were harvested for antibody staining assay.

Detections of CD Marker Expression in hPB-MNCs by Flow Cytometric Assay

Harvested cells were washed twice by PBS and suspended in 100 μ L PBS with 1×10^6 cells. Fluorochrome-conjugated monoclonal antibodies (mAb) were added to each cell suspension (10 μ L mAb in 100 μ L cell suspension)

for labeling and incubated at 4°C for 30 min. In double staining experiments, two types of mAb conjugated with different fluorochromes were both added to cell suspensions. As negative controls, aliquots of cell suspensions were added with the same fluorochrome-conjugated irrelevant isotype-matched mAb. The cells were washed twice by PBS and resuspended in 4% paraformaldehyde for 10 min to fix cells and stored at 4°C for flow cytometric analysis. FACS Calibur (Becton Dickinson, San Jose, CA, USA) was used for cytofluorometric analysis. 20,000 cells were computed and analyzed for each sample. The software for data analysis used WinMDI ver. 2.8 (Windows Multiple Document Interface for Flow Cytometry). Statistical markers were set using the irrelevant isotype-matched controls as reference.

Isolation of Monocytes and T Cells from hPB-MNCs

Using Monocyte Isolation Kit II and Pan T Cell Isolation Kit II, human monocytes and T cells were isolated by depletion of non-monocytes and non-T cells respectively (negative selection). Total hPB-MNCs were suspended in MACS cell separation buffer and incubated with primary labeling reagent which was a cocktail of biotin-conjugated monoclonal antibodies, 10 μ L of biotin-antibody cocktail per 1×10^7 total cells, at 4°C for 10 min. The anti-biotin monoclonal antibodies conjugated with magnetic microbeads as secondary labeling reagents were then added with 20 μ L per 1×10^7 total cells for further labeling at 4°C for 15 min. In between the two labeling steps, no washing was required. After labeling, the cells were washed by MACS cell separation buffer and applied in the separation columns. Columns were put on the magnetic field of a MACS Separator and rinsed by MACS cell separation buffer before sample loading. The unlabeled cells passed through the columns were collected and cultured in RPMI-1640 CM at the density of 2×10^6 cells/mL.

Co-Culture Experiment of Monocytes and T Cells

1×10^6 isolated monocytes and 5×10^5 T cells were seeded in the same well with 750 μ L RPMI CM in the presence of 100 μ M sugiol or equal volume of DMSO as control. After 24-h stimulation, the suspended cells, not the adherent monocytes, were harvested. The harvested cells were then incubated with anti-CD25-PE and anti-CD69-PE antibodies for flow cytometric analysis. T cells were gated by staining with anti-CD3-FITC antibody or by their distinct FSC/SSC properties in flow cytometric assay.

3. RESULTS AND DISCUSSION

Human peripheral blood mononuclear cell (hPB-MNC) was found mostly to be composed of CD3 T cells ($50.21 \pm 5.23\%$), CD19 B cells ($10.01 \pm 3.04\%$), CD14 monocytes ($12.32 \pm 2.09\%$) and CD56 natural killer (NK) cells ($14.03 \pm 3.11\%$) (Fig. 1). The population of hPB-MNCs is occupied by the four cells over 10% which allowed the dynamic changes of CD69 expression of each cell type to be clearly observed. Based on the dynamic changes of CD69 activation makers, cytoxic screening was developed in this study for identifying compounds with immuno-modulating activities from the crude extractions of natural products. The flowchart of cytoxic screening is illustrated in Fig. (2) and its detailed procedures are described as follows. Initially, 1×10^6 hPB-MNCs suspended in 500 μ L culture medium are seeded in

each individual well among 48 multiple-well culture plates. More than forty samples including the natural extracts or identified compounds can be tested on their immunoactivating activities at the same time. After 12 h treatment, the single staining procedures on CD69 monoclonal antibody are performed and then analyzed by flow cytometry. All the procedures of antibody staining are directly handled in the same culture plate. From the primary screening, the samples with the ability to induce CD69 expression are chosen for further assay. The double staining of antibodies is carried out to recognize CD69 and cell type-specific markers so as to engage in a serial purification process to trace the functional components from the extractions of the natural products. Finally, the pure functional compounds are identified by NMR and mass spectra. The immuno-enhancing activities of identified pure compounds were further confirmed by overly expressed CD69 in affected cell populations and in isolated distinct cell populations. As a multiple-parameter screening strategy, cytoxic screening serves as a platform for discovering new drugs and advanced studies on pharmacological mechanisms.

Fig. (1). The distributions of the four cell populations of hPB-MNCs detected for cytoxic screening. Using the monoclonal antibodies recognizing the specific cell surface markers, four cell populations in hPB-MNCs (CD3: T cells; CD19: B cells; CD14: monocytes/macrophages; CD56: natural killer cells) were found. Statistical markers were set using the irrelevant isotype-matched controls as reference. Three independent experiments were performed to determine the mean and SEM.

By single staining with anti-CD69-PE antibody, the ethanol extract of *C. formosana* in hPB-MNCs induced an increase in CD69 expression by 2.5 folds (Fig. 3). PMA and LPS, endotoxins of *E. coli*, which act as strong stimulators for lymphocyte activation, were used as positive controls. Regarding the result of the double antibody staining, the ethanol extract of *C. formosana* was observed to stimulate the increase of CD69 expression in CD3 and CD14 populations of hPB-MNCs by 1.7 and 1.5-fold, respectively (Fig. 4). To track down the functional components, the ethanol extract of *C. formosana* was extracted with *n*-hexane, dichloromethane, ethyl acetate, butanol and deionized water. By double staining, the latter five extracts were further separately examined for their immuno-activating activities in CD3 and CD14 populations. Only the *n*-hexane fraction showed the same immuno-activating properties as the crude extract (data not shown). The *n*-hexane fraction was thus selected for HPLC component separation, and the components with HPLC major peaks were collected for the investi-

Fig. (2). The flowchart of cytomic screening.

gation of immuno-activating activities. A component revealing the specific immuno-activating property was put through the analysis of NMR and mass spectra. The pure functional compound was successfully identified as sugiol. 50mg sugiol was purified from original 205-g ethanol extracts (0.02% yield).

Sugiol is a diterpene and abundant in the bark of *C. formosana*. Previous studies have shown that sugiol exhibited many biological functions, including anti-oxidant [28], anti-

tumor activity on EBV-EA induction [29,30], antiinflammatory [18], antimicrobial [31], and aldose reductase inhibitory activities [32]. In this study, the potential immuno-activating properties of sugiol are discovered by cytomic screening. Fig. (5a) and (5b) demonstrate that sugiol significantly up-regulated CD69 expressions in CD3 and CD14 populations in hPB-MNCs. After sugiol stimulation, the increased distributions of CD3 and CD14 cell populations are illustrated in Fig. (5c). The results suggest that sugiol is able not only to

Fig. (3). The ethanol extract of *C. formosana* induces CD69 expression in hPB-MNCs. 1×10^6 hPB-MNCs cultured in 500 μ L RPMI-CM were treated with PMA (20 ng/mL), LPS (1 μ g/mL) and ethanol extract of *C. formosana* (crude extracts, 100 μ g/mL) respectively for 12 h. hPB-MNCs were singly stained with anti-CD69-PE antibody and analyzed for the percentages of positive signals relative to isotype negative controls. The percentages of hPB-MNCs with positive fluorescence signals were calculated and normalized to the value without treatment. Results are expressed as the mean $\pm$ SEM from three independent experiments.

up-regulate the CD69 expression but also to stimulate the proliferation of both CD3 and CD 14 cells. More importantly, the results confirm that sugiol is the functional compound, which activate CD69 expression in CD3 T cells, CD14 populations during cytomic screening processes.

CD3 T cell is composed of two subpopulations: CD4 helper T cells and CD8 cytotoxic T cells. On one hand, the CD4 helper T cells play a regulatory role in activating cytotoxic T cells and macrophages to attack pathogen-infected cells or to stimulate B cells to secrete antibodies. On the other hand, the CD8 cytotoxic T cell are capable of generating the death of infected or tumorous cells [33]. To clarify the sugiol-affected subpopulations in CD3 T cells, CD4 and CD8 markers were introduced to indicate CD69 expressions. We found that sugiol raised the activation and

expansion in the CD8 cytotoxic T cell, while the CD4 helper T cells were not apparently affected (Fig. 6). The results indicate that the activation of CD3 T cells observed in hPB-MNCs may be resulted from the activated CD8 subpopulation. Moreover, cytomic screening with multiple-parameter characterization provides effective markers for specific observations from a different perspective.

In the physiological environment, CD14 monocyte activation generally enhances the antigen-presenting capacity and the phagocytosis of infected components. In addition, to regulate the immune functions in other cells, CD14 monocytes modulate the patterns of cytokine secretion and the expression of functional adhesion molecules. Hence, the cross-talks among different cell populations in sugiol-treated

Fig. (4). The ethanol extract of *C. formosana* specifically induces CD69 expression in CD3 and CD14 cell populations in hPB-MNCs. Double staining using the monoclonal antibodies for specific cell type markers (CD3: T cells; CD19: B cells; CD14: monocytes/macrophages; CD56: natural killer cells), was performed to reveal the affected hPB-MNC cell populations after 24 h treating with the ethanol extract of *C. formosana* (the gray columns of crude extracts, 100 μ g/mL). The double positive cells were calculated and normalized to that of the control experiment (without treatment), and the results are expressed as the mean $\pm$ SEM from three independent experiments.

hPB-MNCs were considered. By magnetic separations, we purified CD 14 and CD3 cell populations to assess the direct or indirect effects of sugiol on monocytes and T cells. After treating the two distinct cell populations separately with sugiol for 24 h, the expression of CD69 in monocytes was increased by 3.5-fold (from 9.12% to 32.65%). Moreover,

CD80 and CD86, known as co-stimulatory molecules for T cell activation and served as markers for activated antigen-presenting cells [34], also increased in expressions (CD80: 8.91% to 31.03%, CD86: 56.21% to 74.09%) (Fig. 7a). In contrast, we found that sugiol had no effect on the phenotypes of isolated CD3 T cells because the expressions of CD69 and CD25 (lymphocyte specific activation marker) were not induced (Fig. 7b). The results show that CD14 monocytes were directly activated by sugiol. However, CD69 expression in CD3 T cells was only activated by sugiol stimulations in hPB-MNCs, but not in isolated cell culture. This suggests that the activation of the CD3 T cells may be caused by factors provided by other cell populations in hPB-MNCs.

The explanation for the indirect effect of sugiol-treatment in CD3 T cells may be that through CD14 monocyte activation, cytokines and co-stimulatory molecules were expressed and participated in T cell induction [35]. To verify this hypothesis, we co-cultured CD14 monocytes and CD3 T cells and treated the co-culture with sugiol. By gating CD3 positive cells in flow cytometric analysis, we found that the expressions of CD69 and CD25 were induced in T cells (Fig. 8). This phenomenon provides the evidence for the indirectly activated mechanisms of sugiol in CD3 T cells. However, the level of T cell activation was not as strong as that in hPB-MNCs. Therefore, other cell populations may be involved in the networks of indirect mechanisms of sugiol-induced T cell activations. Possible candidates include the dendritic cells and other cell types in hPB-MNCs.

Fig. (5). The immuno-activating properties of Sugiol in hPB-MNCs. (a,b) Sugiol specific induction of CD69 expressions in CD14 and CD3 cell populations. Double staining was performed in hPB-MNCs treated with sugiol (100 μ M) after 24 h and cells treated with equal volume of DMSO were used as control (without treatment). The percentages of CD14⁺/CD69⁺ and CD3⁺/CD69⁺ cells were measured and normalized to the value without treatment. (c) Sugiol increased CD14 and CD3 populations in hPB-MNCs. The positive regions of X-axis were used to calculate the percentages of CD14⁺ and CD3⁺ cells and the values were normalized to that without treatment. Results are expressed as the mean $\pm$ SEM from three independent experiments.

A lot of natural extracts and medicine modulate immune functions with diverse targets and levels that cause the com-

plexity of bioactive assay. This study demonstrates the feasibility of using cytotoxic screening as a multiple-parameter screening strategy to identify compounds with immunomodulating properties from the crude extracts of natural

products. Combinatorial CD marker detection by flow cytometry enhances the performance of high throughput screening and reveals additional functions in the immune modulation properties of tested samples. Here we performed the cytomic screening based on the dynamically expressed changes of CD69 activation marker among four major distributed cell populations in the heterogeneous mixture of immune cells. Paralleling the purification steps from the crude extracts of *C. formosana*, cytomic screening enabled us to trace and identify the functional components. The results demonstrate that a single compound, sugiol, exhibited the major bioactivities.

Fig. (8). Co-culturing CD3 T cells and CD14 monocytes induces the activation of CD3 T cells. In the presence of sugiol (100 μM), CD3 T cells and CD14 monocytes were co-cultured at 2:1 ratio for 24 h. The cells were harvested for CD69 and CD25-antibodies staining and T cell population was gated by CD3 positive signals. The cells were analyzed and the percentages of positive cells relative to isotype negative controls were determined. Results are expressed as the mean ± SEM from three independent experiments.

An important advantage of cytomic screening is that it introduces meaningful CD markers with multiple-parameter characterization which makes a distinct way of observation undertaken. During cytomic screening processes, CD4 and CD8, the markers of T cell subpopulations, were introduced to clarify that sugiol-affected CD3 T cell activation was in CD8 cytotoxic T cell subpopulation but not in CD4 helper T cell subpopulation. Furthermore, to assess the direct or indirect effects of sugiol on isolated monocytes and T cells, CD 80, CD86 and CD25 were introduced. Sugiol was found to activate CD69, CD80, and CD 86 expressions in isolated monocytes, whereas sugiol showed no direct effect on isolated T cells because the expressions of CD69 and CD 25 were not induced. And yet, through the up-regulated expressions of CD69 and CD25 in co-cultured experiment, the indirectly activated mechanisms of sugiol in CD3 T cells were verified.

These results provide more precise information for further development of immunomodulating medicine. Sugiol induces the CD3 T cell proliferation and activation, especially in CD8 cytotoxic T lymphocytes. This property reveals sugiol may be applied in anti-tumor therapy to enhance the host cellular cytotoxicity against the tumor. Furthermore, sugiol activate CD14 monocytes may induce macrophage maturation and increase antigen-presenting ability in the face

of virus-infected or tumorous cells. Thus, with potential, sugiol may increase the response of the host defense ability as an immunotherapeutic strategy.

The experimental data of this study reveals that cytomic screening with multiple-parameter characterization could serve as a platform for identifying compounds with immunomodulating properties from the crude extracts of natural products, leading to drug discoveries and advanced studies on pharmacological mechanisms.

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ABBREVIATIONS

hPB-MNCs	= Human Peripheral Mononuclear Cells
CD	= Cluster of Differentiation
<i>C. formosana</i>	= <i>Calocedrus formosana</i>
CM	= Complete medium
MACS	= Magnetic Cell Sorting
NK cells	= Natural Killer cells
PMA	= Phorbol 12-myristate 13-acetate

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