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Original Research Article

Antioxidant and Anticancer Potentials and Metabolic Profiling of Benjakul, A Thai Herbal Preparation

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ABSTRACT

Benjakul (BJK), a Thai folk remedy has been used in traditional medicine for the treatment of various illnesses. This study aimed to assess the antioxidant, and anticancer properties, and metabolic profile of Benjakul. The antioxidant activity of the ethanol extract of BJK and its component medicinal plants (*Piper retrofractum* Vahl., *Piper sarmentosum* Roxb., *Piper interuptum* Opiz., *Plumbago indica* L., and *Zingiber officinale* Rosc.) was assessed using the Ferric Reducing Antioxidant Power (FRAP) assay, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The anticancer activity was assessed through cytotoxic effect against cholangiocarcinoma (CCA) (bile duct cancer) cell lines (KKU-213B and KKU-100) using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The metabolic fingerprint and potential bioactive compounds in the ethanol extract of BJK and component medicinal plants was investigated using Nuclear Magnetic Resonance (NMR) spectroscopy. The FRAP and DPPH assays revealed that the ethanol extracts of BJK and its component medicinal plants possessed antioxidant activity with *Zingiber officinale* exhibiting the highest activity. The FRAP value and EC_{50} for DPPH radical scavenging activity for *Zingiber officinale* were 94.860 ± 0.156 µg AAE/mg extract and 86.63 g/mL, respectively. The metabolic profiling identified vanillic acid and curcumin as the main bioactive metabolites in BJK and its component medicinal plants. The partial least squares (PLS) loading plot revealed that the methoxy group is associated with the inhibition of CCA cells, and phenolic hydroxyl protons as the potential pharmacophore of the bioactive molecules. Therefore, BJK could be considered as a potentially effective treatment for bile duct cancer.

***Keywords*:** Benjakul, Thai Traditional medicine, Antioxidant, Anticancer, Metabolic profile.

Introduction

Natural products have played an important role in the discovery of new drugs and their innovative mechanisms of action. Antioxidant effect is one of the most extensively studied properties exhibited by substances derived from natural products.[1] Many plants have been reported as sources of natural antioxidants, and the discovery and use of these naturally occurring antioxidants derived from plant products is beneficial to humankind.

Thai traditional medicine based on natural products especially medicinal plants plays an important role in the treatment of various diseases. The bioactive components and pharmacological effects of these plants are still insufficiently studied.[1]

Phytochemicals with antioxidant properties derived from medicinal plants have been shown to extend the lifespan of animal models.[2] For example, proanthocyanidins and polyphenols extracted from blueberries, protect cells against oxidative stress.[3] A traditional Chinese multi-herbal extract was discovered to produce antioxidant and anti-aging effects.[2]

Benjakul (BJK), a Thai herbal remedy, is a common treatment for inflammation and cancer in Thai traditional medicine and it is included on the National Lists of Essential Medicines. BJK comprises five medicinal plants including *Piper retrofractum* Vahl. (Pr), *Piper sarmentosum* Roxb. (Ps), *Piper interuptum* Opiz. (Pi), *Plumbago indica* L. (Pn), and *Zingiber officinale* Rosc. (Zo). BJK has been reported to be used as anticancer remedies in Thai traditional medicine. It has been used as the initial medication to reduce pain and manage patients' symptoms prior to the administration of other treatments.[4,5] Previous reports have indicated that the herbal remedy and its individual plant components exhibit cytotoxic effects against cancer cells[6] anti-allergic[7]and anti-inflammatory effects.[7–10] In addition, isolated compounds from BJK have shown cytotoxic activity on breast cancer cells,[11] and large and small cell lung cancer cells.[12,13]

It has been demonstrated that plant extracts and plant-derived compounds exhibit anticancer properties by activating the apoptotic pathway.[14–17] Some specific plant components in the BJK formulation caused cell death by apoptotic signalling.[18–21]

At present, nuclear magnetic resonance (NMR) spectroscopy is increasingly being used in the fields of food science,[22–24] medicine and health,[25–29] and natural products.[30] Recently, NMR was used to analyze the Chinese herbal medicine, *Sophora flavescens*, and two novel chemical compounds were identified.[31] In addition, a previous study used NMR-based metabolomics to identify changes in the metabolic profile of a local cultivar of *Andrographis paniculata* during its growth period.[32]

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The purpose of this study was to evaluate the antioxidant and anticancer activities of BJK and identify its bioactive constituents, which could serve as new therapeutic alternatives for the prevention and treatment of cancer.

Materials and Methods

Reagents and chemicals
Unless otherwise stated, the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). The F-12 Nutrient Mixture (HAM) and fetal bovine serum were purchased from Thermo Fisher Scientific (MA, United States).

Plant collection and identification
The BJK remedy is composed of 5 medicinal plant species, *Piper retrofractum* Vahl. (Pr), *Piper sarmentosum* Roxb. (Ps), *Piper interuptum* Opiz. (Pi), *Plumbago indica* L. (Pn), and *Zingiber officinale* Rosc. (Zo). Each medicinal plant was collected in July, 2022 from the northern region of Thailand. The Plant materials were authenticated by Ms. Dujhathai Anekchai, Pharm.D, at the Pharmaceutical Laboratory Service Centre, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The following herbarium no. SKP 146161803, SKP 146161907, SKP 146160902, SKP 148160901 and SKP 206261507 for *Piper retrofractum* Vahl., *Piper sarmentosum* Roxb., *Piper interuptum* Opiz., *Plumbago indica* L., and *Zingiber officinale* Rosc., respectively were issued.

Preparation of extracts
Each herbal plant was dried in an oven at 60ºC, then ground into fine powder. The powdered plant (100 g) was extracted by maceration in 1 L of 95% ethanol at room temperature for 5 days. The ethanol extract was filtered using Whatman filter paper no. 1, and the extract was concentrated using a rotary evaporator (Buchi, Essen, Germany) at 50°C under reduced pressure. The concentrated ethanol extracts were stored at 4°C until used.[33]

Evaluation of antioxidant activity
Ferric Reducing Antioxidant Power (FRAP) assay
The antioxidant capacity of the ethanol extracts was determined by FRAP assay following a slightly modified procedure.[34] The FRAP working solution was freshly prepared by mixing 20 mM ferric chloride ($FeCl_3$) in acetate buffer, 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in acetate buffer and 0.25 mM sodium acetate buffer (pH 3.6) in a 1:1:10 ratio. Each extract was combined with 182 µL of FRAP solution, and the absorbance was measured at 593 nm using a microplate reader. Calibration curve was generated using ascorbic acid at concentrations ranging from 0 to 60 µg/mL. The antioxidant capacity of the extracts was estimated from the equation of the standard curve of ascorbic acid, and the results were expressed as micrograms of ascorbic acid equivalent per milligram of extract (µg AAE/mg extract).

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay
The working reagent was prepared by dissolving DPPH in absolute ethanol at a concentration of 0.1 mM. Twenty microliters of the extract solution was mixed with 180 µL of DPPH solution. The mixture was incubated at room temperature in the dark for 30 min. The absorbance was measured using a spectrophotometer at a wavelength of 517 nm. The DPPH free radical scavenging capacity was determined and compared with standard ascorbic acid. The percentage of radical scavenging effect was calculated by the following equation:

Percentage DPPH radical scavenging effect (%) = (Ab – As)/Ab x 100

Where; Ab is the absorbance of blank (DPPH without extract), and As is the absorbance of the reaction mixture (DPPH with the extract).
The EC_{50} values (the concentration needed to achieve a 50% antioxidant effect) of the extracts was calculated from the plot of percentage radical scavenging activity versus concentration of the extracts.

Evaluation of anticancer activity
Cholangiocarcinoma cell lines
Cholangiocarcinoma (CCA) cell lines; KKU-213B and KKU-100 were purchased from the Japanese Collection of Research Bioresources (JCRB). The CCA cell lines were produced by Prof. Banchob Sripa of Khon Kaen University in Thailand, and both cell lines were deposited at the JCRB, Osaka, Japan, for thorough characterization. The cell lines were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (50 U/mL and 50 g/mL), and then placed in a humidified incubator at 37°C with 5% CO_2. A primary normal fibroblast cell line was purchased from American Type Culture Collection (ATCC). The cell line was cultured in Eagles's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum and penicillin/streptomycin (50 U/mL and 50 µg/mL) and incubated in a humidified incubator of 5% CO_2 at 37°C.

Test for cytotoxic activity
The MTT colorimetric test was utilized to assess the cytotoxic effects of BJK. KKU-213B and KKU-100 cells were placed in a 96-well plate at a density of 2,000 cells per well. Cells were cultured for 24 h and then treated with various concentrations of the crude ethanol extracts for 48 h and 72 h at 37°C in a 5% CO_2 condition. The cells were then rinsed with phosphate buffered saline (PBS) and exposed to MTT reagent at 37°C for 2 h. The MTT reagent was removed, and DMSO was introduced to dissolve the formazan crystals, and the absorbance was measured at 570 nm using micro-plate reader.
The percentage cell viability was calculated using the following formula:

$$\text{Percentage cell viability (\%)} = \frac{\text{Absorbance of untreated cells} - \text{Absorbance of culture medium}}{\text{Absorbance of treated cells} - \text{Absorbance of culture medium}} x\ 100$$

Nuclear magnetic resonance (NMR) acquisition for metabolic profiling
The ethanol extract (100 g) was dissolved in a 1 mL of CD_3OD (with 0.1% v/v tetramethylsilane, TMS). The mixture (600 µL) was placed in a 5-mm NMR tube for NMR analysis. The ^{1}H-NMR spectrum was obtained using a 400 MHz NMR instrument (Avance, Bruker BioSpin GmbH, Rheinstetten, Germany). The zg30 pulse sequence was applied with water signal suppression and 32 scans at 25°C. Following the capture, typical FID processing methods were performed using TopSpin (Bruker, Biospin, Italy), including Fourier transform, phase correction, and baseline correction. All the ^{1}H-NMR spectra were calibrated with reference to the internal standard TMS (δ = 0.00 ppm). Metabolite identification was performed by statistical total correlation spectroscopy (STOCSY) by comparing against metabolome databases such the human metabolome database (HMDB), biological magnetic resonance data bank (BMRD), and natural product magnetic resonance database (NP-MRD).[35,36]

Statistical analysis
Statistical analysis was conducted using IBM SPSS V.23.0 software by SPSS Inc., Chicago, IL. Data were expressed as means ± standard deviation (SD) of three independent experiments. GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA) was used to compute and construct a bar chart for cytotoxic activity and a dose-response curve for EC_{50}. One-way ANOVA was used to determine the statistical significant difference among means. $P < 0.05$ was considered statistically significant.
For NMR data, multivariate data analysis (MVDA) was applied to identify the bioactive metabolites. All spectra from 2.7 were aligned based on a hierarchical cluster-based peak alignment (CluPA) in R statistical software using the ASICS package,[37,38] and normalized by probabilistic quotient normalization method.[39] Then, pareto scaling was applied using the Metabom8 package to all data prior to MVDA including unsupervised principal component analysis (PCA) and supervised partial least squares regression analysis (PLS). The fraction of the variance explained (R^2) by a component, indicates how well the variation of a variable is explained. The goodness of fit prediction (Q^2) was used to determine how well a variable could be predicted and the accuracy of prediction was estimated by cross validation. Quantitative

structure-activity relationships (QSAR) aim to maximize unexplained variation in dependent measures. Q^2 values equal to or greater than 0.4 were considered acceptable (Worley and Powers, 2015).

Results and Discussion

Antioxidant activity

Phyto-antioxidants, which are found in medicinal plants, can actually prevent oxidative stress. Both FRAP assay and DPPH radical scavenging assay were used to assess the antioxidant properties of BJK herbal remedy and its component medicinal plants extracts.

FRAP assay was used to determine the ability of antioxidants present in the herbal plants to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) through the donation of an electron. The correlation coefficient (r) of the calibration curve, plotted using standard ascorbic acid concentrations versus absorbance was greater than 0.95. The results revealed that the ethanol extracts of BJK herbal remedy and its components medicinal plants had antioxidant activity ranging from 26.130 ± 0.962 to 94.860 ± 0.156 µg AAE/mg extract, with the ethanol extract of *Z. officinale* exhibiting the highest activity with a FRAP value of 94.860 ± 0.156 µg AAE/mg extract (Figure 1). However, in a previous study, the FRAP value of ginger juice, was found to be 348.70 µgAAE/mg extract,[41] a value significantly higher than what was obtained in our study. The variation in the FRAP value may be attributed to the differences in the extraction method used.

The DPPH scavenging activity of the extracts was expressed in terms of half maximal effective concentration (EC_{50}). The EC_{50} values of the extracts range from 86.63 to 237.40 µg/mL compared to the standard ascorbic acid (15.10 µg/mL). Lower EC_{50} value indicates higher DPPH radical scavenging activity, hence, a higher antioxidant potential. Overall, the ethanol extract of Zo had the lowest EC_{50} value (86.63 µg/mL) followed by BJK, Pn, Ps, Pi and Pr (Figure 2A). At a concentration of 250 µg/mL, Zo had the highest DPPH radical scavenging activity (Figure 2B). This indicates that the ethanol extract of Zo had the highest antioxidant activity compared to the other extracts.

Phyto-antioxidants, which are found in medicinal plants, can actually prevent oxidative stress, which has been implicated in cancer pathogenesis.[40] In the present study, DPPH radical scavenging and FRAP assays were used to assess the antioxidant properties of BJK remedy and its component medicinal plants extracts. *Z. officinale* (Zo) commonly called ginger exhibited the highest antioxidant activity in both FRAP and DPPH radical scavenging assays. This observation is similar to the findings from previous studies where the extract of *Z. officinale* was found to possess the highest antioxidant activity among other plants investigated.[42,43]

Anticancer activity

CCA patients usually undergo chemotherapy because this cancer is often diagnosed at an advanced stage.[44] Nevertheless, CCA treatment proves difficult due to strong chemoresistance mechanisms,[45] which suggest a limited response to available chemotherapeutic agents. Therefore, this study has identified the herbal remedy Benjakul (BJK) as a new and potentially useful alternative treatment for CCA. The potential of BJK to inhibit the proliferation of KKU-213B and KKU-100 CCA cell lines was determined by treating the cells with the ethanol extracts of BJK and its herbal components for 48 and 72 h. At a concentration of 100 µg/mL, each herbal extract exhibited strong anti-proliferative effect against CCA cell lines, with the most potent anti-proliferative effect induced by Zo, followed by Pn, Pr, BJK, Ps and Pi, in that order (Figure 3A-3D).

The present results demonstrated that the IC_{50} of BJK at 48 and 72 h was 93.61 µg/mL and 76.13 µg/mL for KKU-213B, and 89.94 µg/mL and 80.16 µg/mL for KKU-100 (Figure 4A and 4B). In addition, BJK was found to be non-toxic to normal fibroblast cells at the same concentration as shown in Figure 4C.

BJK exhibited dose-dependent cytotoxic effects on CCA cells while showing lower toxicity towards normal fibroblast cells. BJK has previously been utilized to treat many kinds of cancer to improve the quality of life for patients.[13] According to previously published reports, the difference in the IC_{50} values of BJK for CCA compared to MCF-7 breast cancer and COR-L23 lung cancer cell lines is greater than twofold and threefold, respectively.[11,13]

Metabolic profiling of BJK remedy and its component medicinal plants

All secondary metabolites in ethanol extracts of BJK and its component medicinal plants were examined concurrently using metabolic profiling. A Principal Component Analysis (PCA) transformed the high-dimensional NMR spectral data to a few latent variables, with the first principal component (PC1) accounting for the greatest variation and the second principal component (PC2) accounting for around 85% of the overall variance.

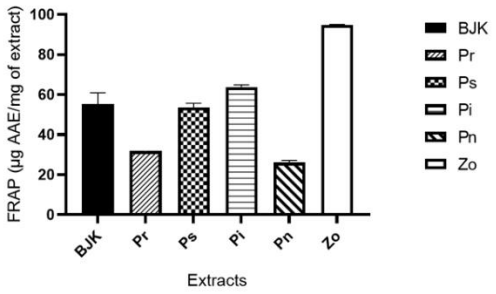

Figure 1: Antioxidant capacity of ethanol extracts of BJK and its component medicinal plants as determined by FRAP assay. Values are presented as mean ± SD of triplicate determination. BJK = Benjakul, Pr = *Piper retrofractum*, Ps = *Piper sarmentosum*, Pi = *Piper interuptum*, Pn = *Plumbago indica*, Zo = *Zingiber officinale*.

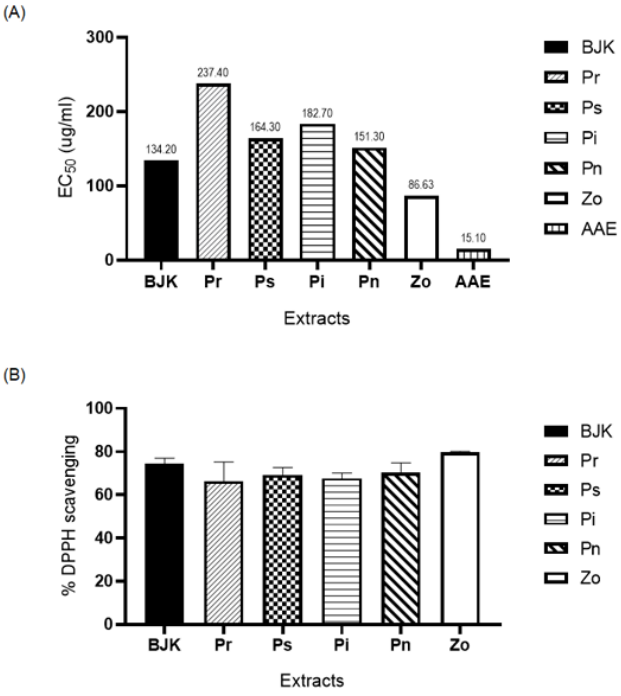

Figure 2: DPPH radical scavenging activity of BJK remedy and its medicinal plants extracts. (A): Half maximal effective concentration (EC_{50}) of the extracts compared to standard ascorbic acid. (B): Percentage scavenging activity of the extracts at 250 µg/mL.
BJK = Benjakul, Pr = *Piper retrofractum*, Ps = *Piper sarmentosum*, Pi = *Piper interuptum*, Pn = *Plumbago indica*, Zo = *Zingiber officinale*.

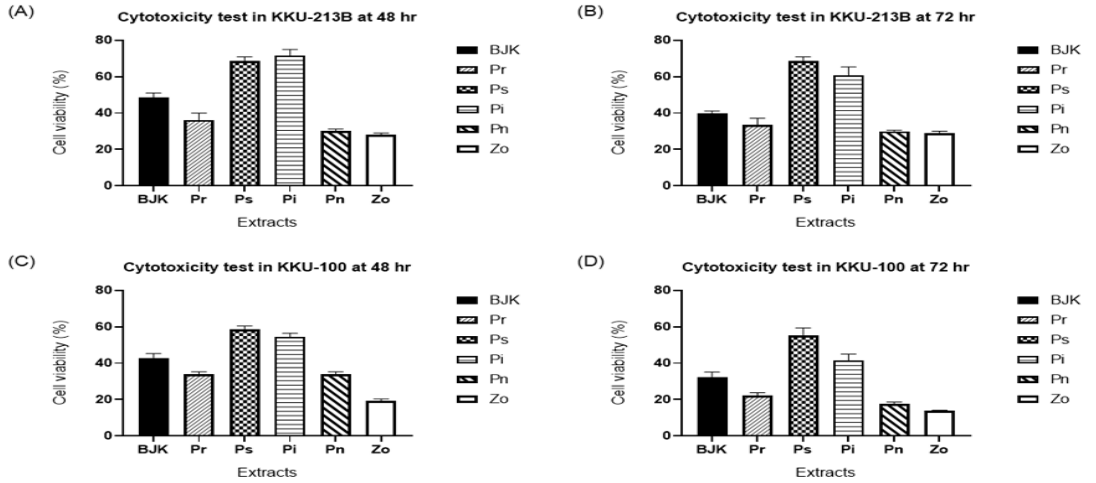

Figure 3: Cytotoxic effects of BJK and its medicinal plant components on CCA cell lines. KKU-213B (A & B) and KKU-100 (C & D) were treated with ethanol extracts of BJK and its herbal components for 48 and 72 h.

The NMR spectra among the herbal extracts were distinguishable due to the differences in their phytochemical constituents (Figure 5). On the negative side of the plot, the score plot demonstrated that Pn was distinct from the other herbal extracts. The CCA cell inhibitory activity of Zo, Pr, and Pn was related to their metabolites. The PLS loading plot showed that the metabolites are positively correlated to the CCA cell inhibitory activity on both KKU-213B and KKU-100. The metabolites assignment of the peak at 3.51 ppm corresponded to methoxy (-OMe) group of vanillin and vanillic acid.[46] Hypoxia-inducible factor 1 (HIF-1) expression suppression has also been demonstrated as a mechanism by which vanillic acid inhibit the metastasis of colon cancer.[47] Vanillic acid has been shown to inhibit lung carcinogenesis by anti-inflammatory and free radical scavenging mechanisms in Swiss albino mice.[48] Vanillin extracted from millet was shown to inhibits the proliferation of HT-29 cells through G0/G1 cell cycle arrest and induction of apoptosis.[49]

The bioactive metabolites in the crude herbal extracts, guided by CCA cell inhibition, were investigated using a partial least squares (PLS) regression analysis. The PLS score plot as shown in Figure 6 revealed a cluster of samples colored by CCA viability after being treated with each crude herbal extract. The cancer cell inhibitory activity was related to the metabolites of Zo, Pr, and Pn. The PLS loading plot showed that the methoxy (-OMe) group was correlated to the cancer cell inhibition of both KKU-213B and KKU-100. Methoxy and hydroxyl groups contribute to the antioxidant activity of phenolic acid through their ability to transfer proton and quench free radicals. Figure 7 shows the partial least squares discriminant analysis (PLS-DA) loading plot of the bioactive compounds. The doublet of doublets (dd) and doublets (d) peaks at 6.44 and 6.65 ppm, respectively, corresponds to the protons of the phenolic hydroxyl group of vanillic acid and curcumin, respectively. Silver nanoparticles of vanillic acid have been developed to improve drug delivery and enhance biological effect of vanillic acid,[50] but the metabolic alterations and health implications continue to pose a challenge. Hence, the biological mechanisms responsible for the anti-CCA effect of vanillic acid need to be studied in detail. This study is the first to show the effect of BJK on CCA cell lines. We believe that BJK herbal remedy could be potential therapy against bile duct cancer.

Conclusion

This study has evaluated the antioxidant, and anticancer activities, and examined the metabolic profile of BJK and its component medicinal plants. The ethanol extracts of BJK and its component medicinal plants possessed antioxidant and cytotoxic activities against cholangiocarcinoma (bile duct cancer) cell lines (KKU-213B and KKU-100), and cytoprotective properties on normal fibroblast cells. Vanillic acid and curcumin were identified as the main bioactive compounds in the plants. The protons of the phenolic hydroxyl group was identifies as the main pharmacophore of the potentially bioactive compounds (vanillic acid and curcumin). Therefore, BJK could be considered as a potentially effective treatment for bile duct cancer.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

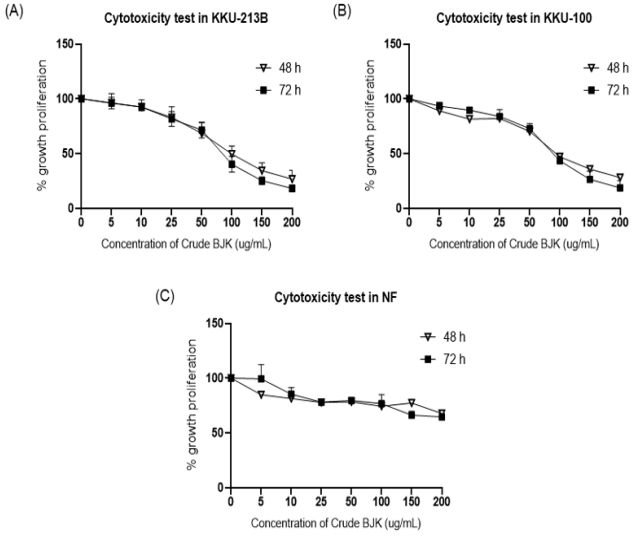

Figure 4: Comparison of the cytotoxic potential of BJK against KKU-213B (A), KKU-100 (B) CCA cell lines and normal fibroblast cells (C) at the same concentration.

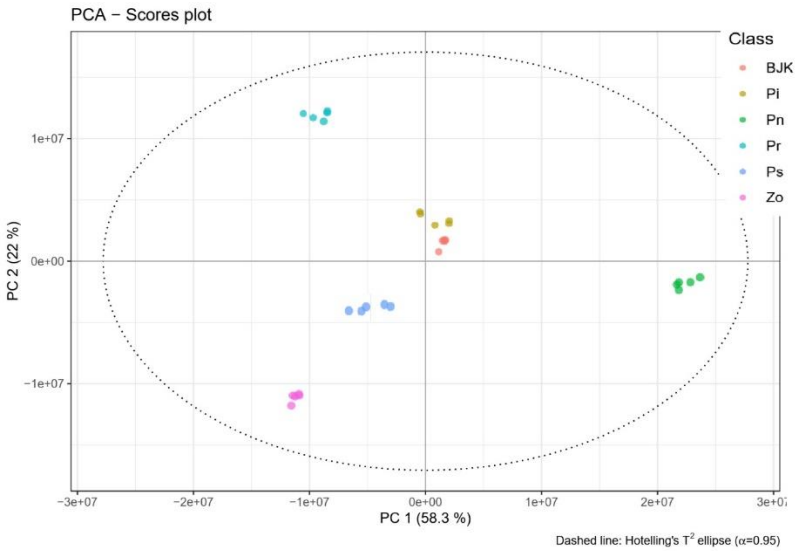

Figure 5: Unsupervised principal component analysis score plot. All phytochemical metabolites in BJK and its component medicinal plant extracts were examined using metabolic profiling. The NMR spectra were distinguishable among the different herbal extracts.

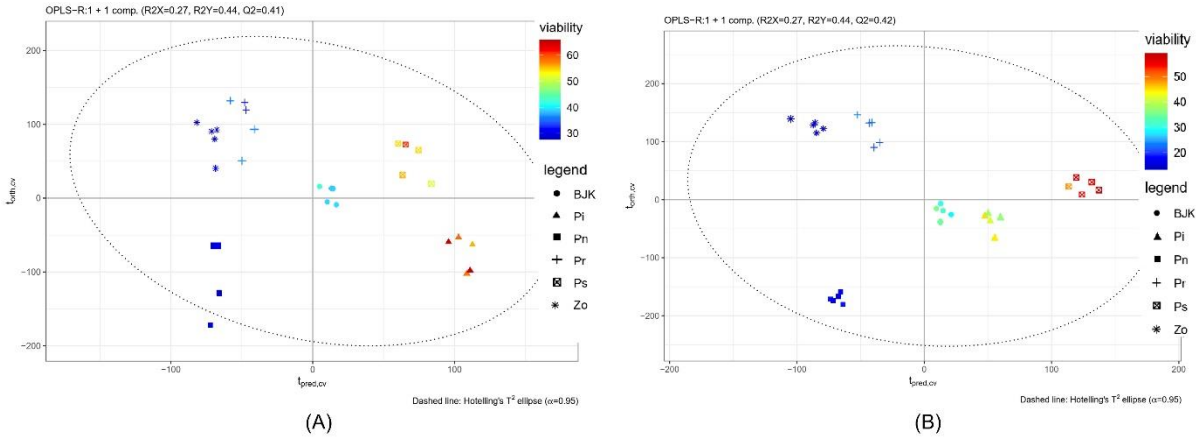

Figure 6: Partial least squares (PLS) regression score plot of crude herbal extract colored by the cell viability; (A): KKU-213B and (B): KKU-100. The PLS score plot revealed a cluster of samples colored by CCA viability after being treated with each crude herbal extract.

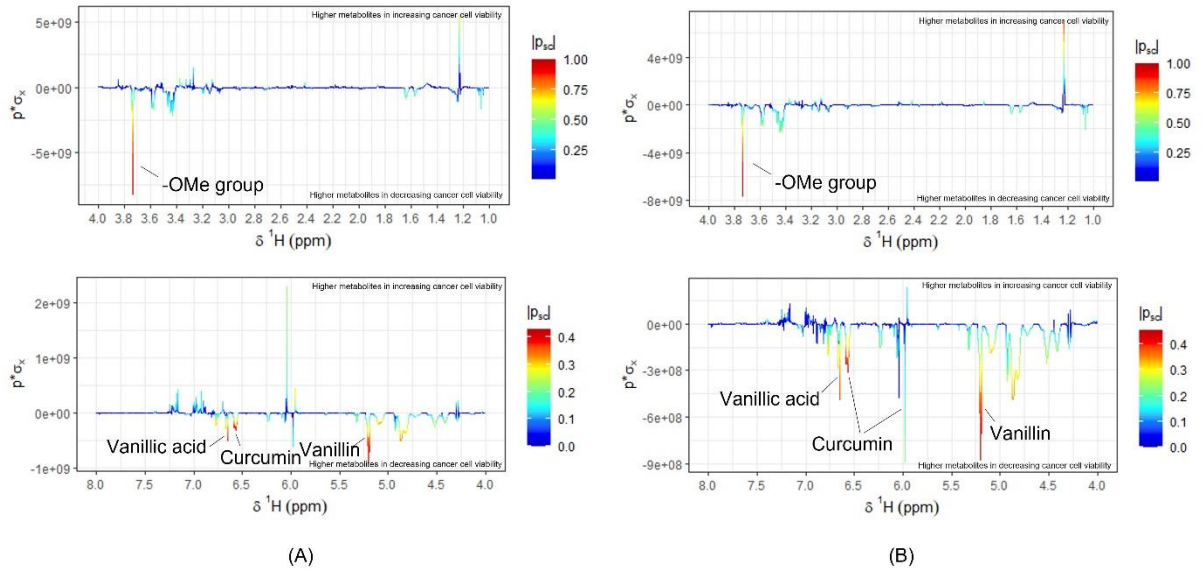

Figure 7: Partial least squares discriminant analysis (PLS-DA) loading plot of bioactive compounds; (A): KKU-213B and (B): KKU-100. The methoxyl and hydroxyl groups in phenolic acids can quench and transfer their proton to free radicals. The doublet of doublets (dd) and doublets (d) at 6.44 and 6.65 ppm, respectively, were the protons in the phenolic hydroxyl groups of vanillic acid and curcumin, respectively.

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