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Journal

BMB Reports, 56(11)

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Publication Date

2023-11-01

DOI

10.5483/BMBRep.2023-0057

Peer reviewed

BMB
Reports

BMB Rep. 2023; 56(11): 594-599
www.bmbreports.org

Discovering the anti-cancer phytochemical rutin against breast cancer through the methodical platform based on traditional medicinal knowledge

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A number of therapeutic drugs have been developed from functional chemicals found in plants. Knowledge of plants used for medicinal purposes has historically been transmitted by word of mouth or through literature. The aim of the present study is to provide a systemic platform for the development of lead compounds against breast cancer based on a traditional medical text. To verify our systematic approach, integrating processes consisted of text mining of traditional medical texts, 3-D virtual docking screening, and *in vitro* and *in vivo* experimental validations were demonstrated. Our text analysis system identified rutin as a specific phytochemical traditionally used for cancer treatment. 3-D virtual screening predicted that rutin could block EGFR signaling. Thus, we validated significant anti-cancer effects of rutin against breast cancer cells through blockade of EGFR signaling pathway *in vitro*. We also demonstrated *in vivo* anti-cancer effects of rutin using the breast cancer recurrence *in vivo* models. In summary, our innovative approach might be proper for discovering new phytochemical lead compounds designing for blockade of malignant neoplasm including breast cancer. [BMB Reports 2023; 56(11): 594-599]

INTRODUCTION

Drug development is a complex process that entails several disciplines in the selection of promising functional chemicals and lead compounds (1). High-throughput screening (HTS) that uses many chemicals synthesized from a pre-designed template is one typical method of identifying target chemicals within the biological area of interest (2). As an alternative, there have been many drugs derived from phytochemicals of plants based on folk remedies, e.g., artemisinin against malaria, tamiflu for swine flu, and most famously, aspirin for relieving pain and reducing fever. Recent approaches for drug discovery have restrictively focused on specific diseases instead of historical records (3).

The study of folk therapy that uses readily available herbs is called ethnopharmacology (4). Dongui-bogam (Treasured Mirror of Eastern Medicine, 1613), a traditional medical classic in Korea, and Bencao-gangmu (Compendium of Materia Medica, 1596), with nearly the same authority and status in China, are the most well-known sources of folk remedies. Both are registered with UNESCO. Dongui-bogam and Bencao-gangmu contain many 'herb treatments' (single herbs) or 'formula prescriptions' (cocktails consisted of several relevant herbs) to treat pathological conditions. Inje-ji collection of benevolent cure is a part of Imwon-gyeongje-ji (collection of practical knowledge in rural life), a huge encyclopedia written by Seo Yugu (1764-1845) compiled in the late Joseon Dynasty in Korea (5). Rutin, a component of many traditional remedies (6), is abundant in buckwheat, apples, black tea, and vegetables. Rutin has been reported to have anti-inflammatory effect, antioxidant effect, and abilities to reduce blood fat and cholesterol (7). Other reports have indicated its anti-cancer effect both *in vitro* and *in vivo* (8).

The aim of the present study is to provide a systemic platform for the development of lead compounds against breast cancer based on a traditional medical text. As one concrete example, we experimentally validated our innovative method to discover a lead compound against breast cancer.

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https://doi.org/10.5483/BMBRep.2023-0057

**Received 14 April 2023, Revised 16 May 2023,
Accepted 21 June 2023, Published online 5 July 2023**

Keywords: 3-D virtual screening, EGFR, *In-silico*, Medical classic, Rutin, Text-mining

ISSN: 1976-670X (electronic edition)

RESULTS

A new systematic approach for identifying specific herbs as potential cancer treatments based on analyses of ethnopharmacological classics

Discovery of medicinal lead compounds was performed through an integrated process that began with text mining and finished with a protein-chemical docking simulation. Phytochemicals in cancer-treating herbs were tested for their interactions with cancer target proteins *in silico*, with phytochemical rutin showing comparable binding affinity to EGFR, an FDA-approved anti-cancer drug. Therefore, rutin was selected for further experimental validation. A functional network analysis of rutin's genes was performed. EGFR-mediated apoptosis and focal adhesion phenotypes were analyzed. Rutin's anti-cancer effects were then tested both *in vitro* and *in vivo*. We compiled treatments specifically targeted to treat cancer from traditional ethnopharmacological classics Dongui-bogam (D) and Inje-ji (IJJ). Ninety-three of 4,247 herbal combined treatments listed in Dongui-bogam were targeted for cancer research. Inje-ji provided 114 combined treatments for cancer out of 3,706 entries. Herbs specifically used for cancer treatments were identified by comparing the number of cancer treatments and all treatments containing herbs (Fisher's exact test with a false discovery rate < 0.05). Twenty-four herbs identified were then sorted and those specifically used for cancer treatment were identified (Table 1).

Because a single herb usually contains many phytochemical ingredients (9), it is difficult to select specific components for further experimental validation of any anti-cancer effects. To circumvent this complexity, we introduced a 3-D virtual docking screening to select promising anti-cancer phytochemicals from those medicinal herbs traditionally used for cancer treatments. To verify our integrated processes for the discovery of anti-

Table 1. Cancer treatment herb specificity table extracted by text mining

Scientific name	Total number of herb cocktails in source text (T)	Number herb cocktails for cancer treatment (C)	Number of herb cocktails containing each herb (H)	Number of herb cocktails for cancer treatment containing each herb (S)	Fisher's exact P	False discovery rate
Citrus reticulata Blanco	4,247	93	216	32	1.11E-16	2.96E-15
Curcuma zedoaria Rosc.	4,247	93	70	30	1.11E-16	2.96E-15
Curcuma zedoaria Rosc.*	3,706	114	109	27	1.11E-16	2.96E-15
Cyperus rotundus Linné	4,247	93	204	29	1.11E-16	2.96E-15
Sparganium stoloniferum Buchanan-Hamilton	4,247	93	85	30	1.11E-16	2.96E-15
Pinellia ternata Breitenbach	4,247	93	512	26	2.18E-05	0.000119412
Croton tiglium L.	4,247	93	70	19	2.22E-16	4.60E-15
Citrus reticulata Blanco.*	4,247	93	676	47	4.22E-15	7.87E-14
Coptis chinensis Franchet	4,247	93	222	28	5.66E-15	9.32E-14
Hordeum vulgare Linné	4,247	93	85	19	6.00E-15	9.32E-14
Poria cocos Wolf	4,247	93	74	18	6.77E-15	9.72E-14
Raphanus sativus L.	4,247	93	19	11	2.00E-14	2.66E-13
Amomum villosum Lour.	4,247	93	138	20	6.23E-12	7.27E-11
Crataegus pinnatifida Bunge (flesh form)	4,247	93	19	9	6.04E-11	6.63E-10
Areca catechu Linné	4,247	93	59	13	2.01E-10	2.09E-09
Magnolia officinalis Rehder et Wilson	4,247	93	255	24	4.36E-10	4.28E-09
Aconitum carmichaeli Debeaux	4,247	93	97	15	1.46E-09	1.36E-08
Ferula asafoetida Linné*	3,706	114	14	8	1.62E-09	1.44E-08
Crataegus pinnatifida Bunge	4,247	93	8	6	2.54E-09	2.15E-08
Rhus verniciflua Stokes	4,247	93	21	8	6.29E-09	5.10E-08
Acorus gramineus Soland.	4,247	93	18	7	5.03E-08	3.61E-07
Zingiber officinale	4,247	93	274	22	5.31E-08	3.67E-07
Gardenia jasminoides Ellis	4,247	93	26	7	9.01E-07	6.01E-06
Aucklandia lappa Decne	3,706	114	390	29	3.43E-06	2.14E-05

T: Dongui-bogam's total number of prescriptions 4,247 and Inje-ji's total number of prescriptions 3,706. All of them are formal herb cocktails consisting of three or more herbs. *C: Number of formal herb cocktails used for cancer treatment. *H: The number of formal herb cocktails including the corresponding herb in each text. *S: The number of formal herb cocktails that have the effect of treating cancer in each text and contain the corresponding herb at the same time.

cancer lead compounds, we performed 3-D virtual screening of 790 phytochemicals in identified cancer-specific medicinal herbs to determine if any of them might be effective against well-known oncogenic drug target protein EGFR (10, 11). Rutin known to be present in *Crataegus pinnatifida Bunge* was selected among sixty-one candidates as a potential phytochemical inhibitor against EGFR since it was calculated to have a considerable binding affinity in the same binding pocket of EGFR as FDA-approved small molecule kinases, erlotinib and gefitinib (12). Rutin has a −8.5 kcal/mol binding affinity to a particular spot on EGFR (co-complexed PDB structure: 4HJO), similar to that of erlotinib (−8.4 kcal/mol). The binding affinity of gefinitib to EGFR (4WKQ) was −8.6 kcal/mol, while rutin's binding affinity was −8.8kcal/mol at the same location of EGFR (Fig. 1A). Moreover, rutin was a splendid candidate for validating this systematic approach, especially when we consider that it is commonly found in *Crataegus pinnatifida Bunge*, *Crataegus pinnatifida Bunge*, *Magnolia officinalis Rehder et Wilson*, and *Gardenia jasminoides Ellis* with statistical significance in cancer treatment cocktails calculated by Fisher's exact test (Fig. 1B). To understand rutin's effects on cancer cells, we performed a functional network analysis with genes related to rutin treatment. Genes (n = 140) associated with physical interaction or transcriptional control among other criteria were selected using a comparative toxicogenomics database (CTD). We performed a protein-protein interaction (PPI) analysis with 36 genes after eliminating non-homo sapiens genes. We then identified various functional groups in the PPI network. After functional enrichment, nine representative pathways were selected by rutin treatment based on the ratio of ProteinsFromNetwork/NumberOfProteinsInGeneSet and manual judgement of importance (Fig. 1C). Apoptosis and focal adhesion were focused on as enriched functional groups induced by rutin treatment in the network. CTD, *BCL2*, *JUN*, *MAPK1*, and *MAPK3* were inhibited by rutin treatment. They are commonly involved in both apoptosis and focal adhesion functional groups. Rutin potentially inhibited *VEGF-A*, *IGF1R*, and *EGFR* in focal adhesion, whereas it activated pro-apoptosis factors *BAX*, *CASP3*, and *CASP7* (Fig. 1D). A network analysis of rutin-target genes based on the number of edges and betweenness centrality revealed that *BCL2*, *JUN*, *MAPK1*, *MAPK3*, *CAS3*, and *EGFR* were critical, although MAPK1 and MAPK3 had relatively lower betweenness centrality than other genes. Notably, EGFR and JUN had enough number of edges and high betweenness centrality (EGFR: 12 and 0.789; JUN: 17 and 0.800, respectively) (Data not shown).

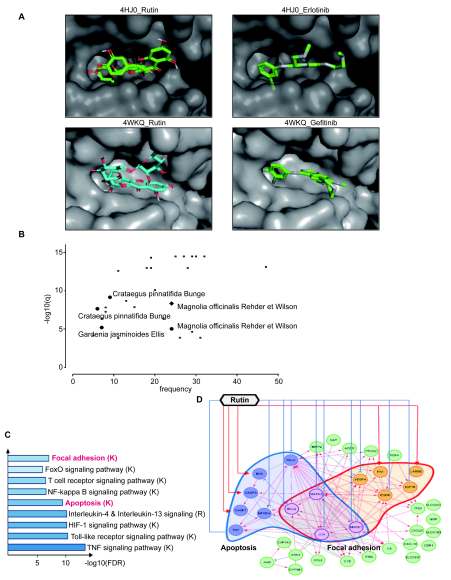

Fig. 1. Selection of rutin using 3-D docking screening against EGFR. (A) Binding affinity of rutin or erlotinib/gefitinib in 4HJO or 4WKQ pocket of EGFR. (B) Frequency of rutin in cancer treatment cocktails. X-axis indicates the number of cancer treatments containing each herb. Y-axis indicates statistical significance of cancer specificity of herbs calculated by Fisher's exact test. Closed circles and diamonds represent herbs that contain rutin. (C) Nine representative mechanisms of Pathway Enrichment Analysis identified by the Reactome Functional Interaction (FI) plugin app of Cytoscape. Red highlighted pathways were functionally defined as Apoptosis and Focal adhesion pathways among the nine representatives. (D) Protein-Protein Interaction (PPI) network among proteins affected by rutin. Nodes with blue and green, and purple background were included in apoptosis (K), focal adhesion (K), and both pathways, respectively. Edges with blunt and arrow indicate inhibition (decreased expression) and activation (increased expression), respectively.

In vitro anti-cancer effect of rutin against human breast cancer cells

To verify biological function of the selected compound using a systematic approach, we examined rutin's ability to inhibit malignant human breast cancer cells. Different human breast cancer cells, MCF-7 and MDA-MB-231, and human embryonic kidney HEK-293T cells, were exposed to different doses of rutin. Cell migration, invasion, adhesion, and proliferation were then assessed to verify any anti-cancer effects. Rutin (0, 0.3, 0.5, 1 μM) inhibited cellular migration of MDA-MB-231 and MCF-7 cells, but not HEK-293T cells, in a dose-dependent manner (Fig. 2A). Treatment with rutin (0, 0.3, 0.5, 1 μM) also significantly decreased cellular invasion of MDA-MB-231 and MCF-7 cells in a dose-dependent manner (Fig. 2B). It also dose-dependently (0, 0.3, 0.5, 1 μM) inhibited the adhesion ability of MDA-MB-231 and MCF-7 cells. However, rutin had no significant effect on HEK-293T cells (Fig. 2C). We tested anti-proliferative effects of rutin using the same conditions. Treatment with rutin (0, 1, 3, 5, 10 μM) showed no effects on the viability of MDA-MB-231 or MCF-7 cells under normal cultured conditions even when its concentration was increased up to 10 μM (data not shown). However, treatment with 10 μM of rutin did marginally decrease the viability of MDA-MB-231 and MCF-7 cells under serum-free cultured conditions (Fig. 2D). Then, we examined signal transduction pathways of receptor tyrosine kinase to

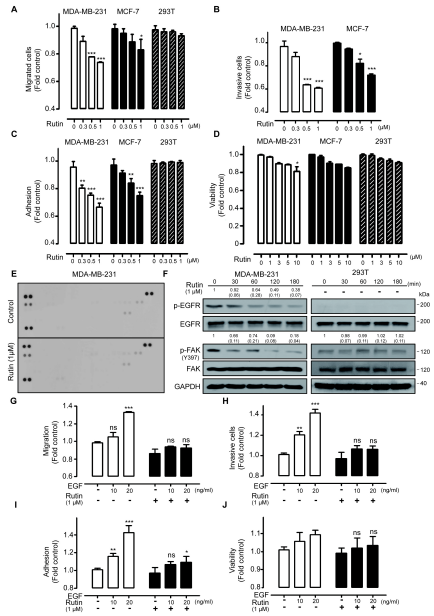

Fig. 2. Sensitivity of human breast cancer cells to rutin. (A) MDA-MB-231, MCF-7, and HEK293T cells were incubated with various concentrations of rutin for 6 h. Migration was evaluated using a Transwell migration assay. (B) MDA-MB-231 and MCF-7 cells were incubated with various concentrations of rutin for 24 h. Invasion was evaluated using the Transwell invasion assay. (C) MDA-MB-231, MCF-7, and HEK293T cells were incubated with various concentrations of rutin for 1 h. Adhesion was evaluated using a fibronectin coated 24-well plate. (D) MDA-MB-231, MCF-7, and HEK293T cells were incubated with various concentrations of rutin for 72 h under a serum-free culture condition. Viability was measured by the WST-1 assay (for migration, invasion, adhesion, and viability assay, $n = 3$; Tukey's *post hoc* test was applied to significant group effects in ANOVA, $P < 0.0001$; asterisks indicate a significant difference compared with 0% inhibition, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., non-significant). (E) MDA-MB-231 cells were incubated with rutin (1 μM) for 24 h. A phospho-RTK array was used to determine differences between rutin-treated and control human breast cancer cells. Data are representative of two individual experiments. (F) MDA-MB-231 and HEK293T cells were incubated with rutin (1 μM) for various time periods and cell lysates were subjected to Western blot analysis using antibodies specific for phospho-EGFR, EGFR, phospho-FAK, FAK, and GAPDH. Relative pixel intensities were measured by densitometry analysis using ImageJ analysis software. Data are representative of three individual experiments. Bracket indicates variance. (G) MDA-MB-231 cells were pretreated with rutin (1 μM) for 24 h. Various concentrations of EGF were treated for 6 h. Migrated cells were evaluated using the Transwell-assay. (H) MDA-MB-231 cells were pretreated with rutin (1 μM) for 24 h. Various concentrations of EGF were used for treatment for 24 h. Invasive cells were evaluated using the Transwell-assay. (I) MDA-MB-231cells were pretreated with rutin (1 μM) for 24 h. Various concentrations of EGF were used for treatment for 1 h. Adhesion was evaluated using a fibronectin coated 24-well plate. (J) MDA-MB-231cells were pretreated with rutin (1 μM) for 24 h. Various concentrations of EGF were treated for 72 h under serum-free cultured condition. Viability was measured by the WST-1 assay (for migration, invasion, adhesion, and viability assay, $n = 3$; Tukey's *post hoc* test was applied to significant group effects in ANOVA, $P < 0.0001$; asterisks indicate a significant difference compared with 0% inhibition, $^{*}P < 0.05$, $^{**}P < 0.01$, n.s., non-significant).

verify the mechanism by which rutin could modulate EGFR signaling. Treatment with exogenous rutin (1 μM) decreased EGFR phosphorylation in MDA-MB-231 cells (Fig. 2E). However, treatment with rutin had no effect on HEK293T cells (data not shown). To further analyze the mechanism, levels of phosphorylated EGFR and FAK were examined. Exogenous rutin treatment dramatically decreased phosphorylation levels of EGFR and FAK in a dose-dependent manner in MDA-MB-231, but not in HEK293T cells (Fig. 2F). We then verified the action mechanism of rutin designating a competition with EGF, a ligand of EGFR. Treatment with different dosages of EGF (10 and 20 ng/ml) significantly increased migration of MDA-MB-231 cells, an effect that was effectively suppressed by pretreatment with a sub-lethal dose of rutin (1 μM) (Fig. 2G). Similar results were obtained using invasion or adhesion assays of MDA-MB-231 cells (Fig. 2H, I). Treatment with 1 μM of rutin also decreased EGF-stimulated viability of MDA-MB-231 cells under serum-starved cultured conditions, although the effect was not statistically significant (Fig. 2J).

In vivo effect of rutin against human breast cancer

To test the inhibitory effect rutin (30 mg/kg), we established recurrence-mimic xenograft modes using MDA-MB-231 cells (Fig. 3A). *In vivo*, an anti-relapse effect was observed in the MDA-MB-231 xenograft model for 30 days following an initial reinjection of primary cultured MDA-MB-231 cells with rutin. PBS-mixed primary MDA-MB-231 cells formed xenograft tumors at a 75% rate (6/8), while rutin-mixed primary MDA-MB-231 cells formed xenograft tumors only at a 10% rate (1/10) (Fig. 3B). Moreover, PBS-mixed primary MDA-MB-231 xenograft tumors had grown to an average size of 168.26 ± 61.71 mm^3 at 30 days after transplantation, while the single rutin-mixed primary MDA-MB-231 xenograft tumor had grown to 95.49 mm^3 (Fig. 3C). There was no significant weight loss in either the PBS or rutin-mixed primary MDA-MB-231 xenograft model (Fig. 3D). To ascertain the anti-relapse effect of rutin at the molecular level, we investigated phosphorylated levels of EGFR and FAK in both PBS and rutin-mixed primary MDA-MB-231 xenograft models. Phosphorylation levels of both EGFR and FAK were significantly decreased in the rutin-mixed primary MDA-MB-231 xenograft tumor sample compared to those in PBS mixed primary MDA-MB-231 xenograft tumor samples (Fig. 3E).

DISCUSSION

Simplified and tidy information of proper chemicals in herbs were retrieved with our text mining approach. Based on these candidates, *in silico* screening revealed that rutin had a binding affinity to a reference position of EGFR, a representative oncogene, comparable to those of FDA-approved anti-cancer drugs, erlotinib and gefitinib. The present hybrid-bioinformatics study provides a rationale for establishing a platform of promising therapeutic lead compound pool based on vast historical experiences of clinical outcomes. Information related to

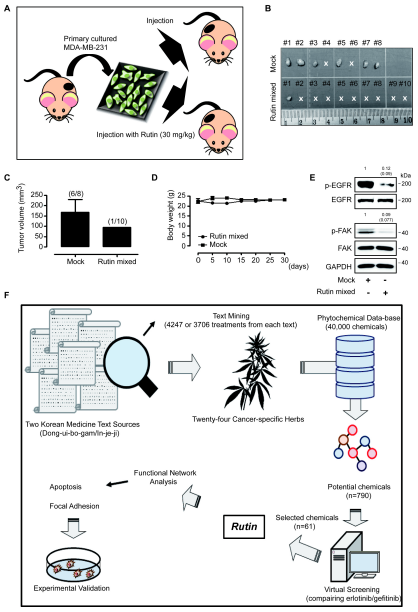

Fig. 3. *In vivo* effects of rutin in breast cancer models. (A) Schematic illustration of rutin-treated *in vivo* experiment. (B) Effects of rutin (10 mg/kg) mixture with primary cultured MDA-MB-231 xenograft models (control group: n = 8, rutin-mixed group: n = 10) were maintained for 30 days. X-mark indicates a lack of tumor formation in each group. (C) Effects of rutin (10 mg/kg) mixture with primary cultured MDA-MB-231 xenograft models (control group: n = 8, rutin-mixed group: n = 10) were measured for 30 days using the following formula: V = 0.523 LW^2 (L = length, W = width) (Statistical analysis was deferred due to the number of tumors formed in rutin-mixed primary-cultured NDA-MB-231 cells injected group). (D) Body weight in each group was measured regularly. (E) Western blot analysis of control and rutin-mixed tumor lysates using anti- phospho-EGFR, phospho-FAK, and phospho-Jun antibodies. GAPDH was used as a loading control. Relative pixel intensities were measured by densitometry using ImageJ software. Western blot analysis data are representative of three individual experiments. Bracket indicates variance. (F) Graphical abstract showing the selection of anti-cancer lead compounds using integrating processes based on text mining.

symptoms and treatments were taken from the two Korean medical classics and the text was mined through a series of computational processes. We extracted promising herb treatments for curing cancers or cancer-like symptoms (also called 'mass'), including treatment with *Coptis chinensis Franchet*, *Curcuma zedoaria Rosc.*, *Ferula asafetida Linne'*, *Magnolia offcinalis Rehder et Wilson*, and *Sparganium stoloniferum Buchanan-Hamilton*, implying that therapeutic ingredients could be selected from dependable information source.

The development of new anti-cancer drugs is one of the focuses of research programs throughout the world. Plants are relatively preferred sources of lead compounds. Many natural product-originated chemicals have been validated as possessing potential anti-cancer properties (13). High-throughput screening (HTS) can test chemicals selected from a set, *i.e.*, a chemical library, depending on specific properties such as drug-likeness, natural product-likeness, and so on. The scale of a natural products chemical library is relatively smaller than that of a synthetic chemical library due to the process of synthetic method of an alternative splicing using template backbone (9). Although HTS can be sued for measuring activities of compounds *in vitro*, safety of compounds is still a critical issue. While chemicals in medicinal herbs, including phytochemicals, have been recognized safer than synthetic chemicals, it remains difficult to extract enough phytochemicals to perform an experiment. In the present study, rutin known to be present in *Crataegus pinnatifida Bunge*, *Magnolia officinalis Rehder et Wilson*, and *Gardenia jasminoides Ellis* was used to validate a well-defined integrative process for drug discovery (6). As predicted, rutin showed *in vitro* anti-cancer properties via blockade of EGFR signaling. Of interested, rutin specifically inhibited the EGFR signaling among 42 receptor tyrosin kinases. Notably, rutin was shown to possess significant *in vivo* anti-recurrence characteristics against breast cancer cells. Breast cancer remains the most prevalent cause of cancer-related deaths in women and approximately 40% of all patients with breast cancer show its recurrence (14), implying that rutin has promising potential against breast cancer relapse based on the impact further study. Further study should be performed using another novel anti-cancer reagent to prove the usefulness of the present systematic approach.

Collectively, our innovative systematic process served as a bridge between past and future cancer treatments. To the best of our knowledge, this is the first report in which texts of ethnopharmacological sources were mined for anti-cancer therapeutic data. We anticipate that this approach will continue to yield promising anti-cancer strategies.

MATERIALS AND METHODS

Text mining

Cancer-specific treating herbs were identified by statistical analysis of decoctions listed in classic Korean medicine texts Dongui-bogam and Inje-ji. Treatments were transformed to a consistent format for computational analysis, then tagged items (words) as either herb or symptom. The relationship between an herb and a symptom can be measured by chi-square test or Fisher's exact P-value. Among total treatments recorded in the text (T), the number of treatments containing an herb used for a specific symptom (C) (cancer in our case) was used for Fisher's exact p-value where the number of treatments containing herbs regardless of symptoms to which the treatment was for (H) and that of treatments for cancer (S) were also used as follows:

$$\textit{Fisher's exact P value} = 1 - \sum_{k=0}^{c} \frac{\binom{S}{K}\binom{T-S}{H-K}}{\binom{T}{H}}$$

3-D docking

Autodock vina software was used to calculate binding affinity between a chemical and a protein as guidance of instructor. To allow for a more configurable position of chemicals in the binding pocket, we enlarged the cubic to 110% of its original size.

Network analysis of rutin-affected proteins

Genes associated with chemicals were identified using Comparative Toxicogenomics Database (CTD). Among 140 genes that could interact with Rutin (last update: 01/06/2020). We used the Reactome Functional Interaction (FI) plugin app of Cytoscape to depict the Protein-Protein Interaction (PPI) network and to perform pathway enrichment and network analysis. We performed functional enrichment with Pathway Enrichment, GO Cell Component, GO Biological Process, and GO Molecular Function with the Reactome plug-in.

ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program of the Korean National Research Foundation (NRF) and funded by the Ministry of Education (2016R1A6A1A030 12862).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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