

Original Research Article

In silico investigation of the mechanism of action of kojic acid effects via protein-protein interaction network

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Abstract

Purpose: To evaluate the molecular mechanism of kojic acid by network pharmacology.

Methods: This study was conducted by designing a protein-protein interaction network through the STITCH database and analyzing biological processes via Cytoscape plugin ClueGO.

Results: A total of 19 protein targets of kojic acid including TYR, NOS3, NOS2, and NOS1 were found. The PPI network helped to understand the mode of action of kojic acid at a molecular level. Gene Ontology (GO) analysis resulted in the retrieval of 104 GO terms which were related to various physiological processes. GO analysis revealed that kojic acid might be involved in the regulation of several biological processes such as circadian gene expression and transcription initiation of RNA polymerase 2.

Conclusion: The findings from this study reveal that the retrieved GO pathways are known to be involved in several diseases such as inflammation, cancer, aging, pigmentation, and melisma. Furthermore, these pathways are directly or indirectly related to kojic acid. Thus, this study has contributed to a better understanding of the mode of action of kojic acid.

Keywords: Network pharmacology, STITCH database, Gene ontology, Circadian regulation, Transcription

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INTRODUCTION

2-Hydroxymethyl-5-hydroxy- γ -pyrone, known as kojic acid, is isolated from fungus *Aspergillus oryzae* and from boiled rice [1]. It has a wide range of applications especially in cosmetics due to its distinguishing whitening feature [2]. Kojic acid exhibits its effect by inhibiting tyrosinase which is an enzyme that converts tyrosine to melanin [3].

Kojic acid comprises a heterocyclic, oxygen-containing structure and it is involved in numerous reactions such as oxidation, reduction, acylation, substitution, and chelation. Due to its potential reactivity, it is used in cosmeceutical preparations [4]. However, the molecular targets and their mechanism of action are still unknown. Therefore, more studies are required to evaluate its therapeutic mode of action and the molecular targets of kojic acid.

In drug discovery, the use of network

pharmacology is very extensive because of its widespread applications for assessing the constituents and analyzing the mechanism of therapeutic effects. Thus, network pharmacology is an approach that speeds up the drug discovery process as well as validates drug actions [5].

The protein-protein interaction network is composed of a variety of proteins which are connected through cellular pathways and play their role in the performance of various activities. It could be hypothesized that the biological activity of kojic acid depends on various protein interactions and molecular pathways [6]. Thereafter, gene ontology (GO) enrichment analysis was also used in this study to genetically annotate the retrieved gene sets [7].

The main objective of this study was to determine the molecular mechanism underlying the effects of kojic acid using network pharmacology. The findings of this study could be presented as a reference for carrying out the clinical trials on kojic acid and its analogs. To conclude this study, GO enrichment analysis of the protein-protein interaction network was conducted by using Cytoscape® software.

METHODS

Network pharmacology gathers information from different sources such as computational and experimental sources. For its illustration, STITCH 5.0 database (<http://stitch.embl.de/>) is used to extract the protein targets and develop a protein-protein interaction network. This database along with other network pharmacological approaches has been used to explore the mechanism of action of Kojic acid. For this purpose, the STITCH database was applied to design a network after extracting protein targets. Secondly, network construction was done using Cytoscape® and its plugin ClueGO to annotate genes.

Retrieval of protein targets

STITCH 5.0 database (<http://stitch.embl.de/>) was used for the retrieval of protein targets of Kojic acid. This database gathers information from various sources via experimental validation, downloadable database, text mining and genome studies to develop a PPI network [8]. Thus, STITCH database designs an interactive network that plots the interactive capability of protein targets with its probabilistic confidence score [9]. In this case, the confidence score was set at 0.150, while the number of interactors was not more than 20 to construct PPI.

Protein interaction network

Cytoscape® (version 2.8.2; <http://www.cytoscape.org/>) is a Java-based software used for analysis of the molecular interactions in PPIN and has an option of using various plug-ins [10]. The technique of graph union operation was adopted to join the small individual networks for the construction of complex PPIN. This software helps to avoid duplication by merging edges and graphs and the reciprocal interaction finding was used to achieve self-loop. A similar type of predictive treatment was adopted for this study.

Topological analysis of network

The topological analysis technique is considered as a valuable strategy to analyze protein interaction networks [11]. It is a powerful tool that can evaluate the values of various parameters such as the number of nodes, diameter and radius of a network, clustering coefficient, total self-loops, and network heterogeneity using network analyzer in Cytoscape [12].

Analysis of biological processes

The biological aspects of target genes and the pathways associated with kojic acid were analyzed through Cytoscape plug-in ClueGO [13]. It is a productive parameter for analyzing gene functions and their statistics. GO enrichment analysis utilized the two-sided hypergeometric test followed by Bonferroni correction for functional visualization of the network. For a medium type network, the significance level was set at $P < 0.05$.

RESULTS

Retrieved of protein targets

A protein-protein interaction network (Figure 1) comprising the retrieved 19 protein targets of Kojic acid was developed using the STITCH database (assessed in Nov 2019). The set confidence score of PPIN was 0.150. According to literature, these retrieved protein targets are vital for play a vital role in establishing pathological, pharmacological and physiological parameters. The information regarding protein targets was further elaborated based on genes-related protein targets by applying Cytoscape plugin ClueGO which is widely used for annotating biological functions. Thus the GO enrichment analysis found 104 GO terms which were categorized into 11 subgroups. Three important GO terms were positive regulation of keratinocyte differentiation, retinoic acid receptor signaling pathway, and positive regulation of autophagy with GO IDs of 0045618, 0048384,

and 0010508 respectively. The genes linked to these analyses were MED1, NCOA3, VDR, and TRIM16 (for 0045618); ACTN4, KMT2E, RARA, RARG, RXRA and TRIM16 (for 0048384); and ATG7, BNIP3L, MEFV, SQSTM1, TBC1D5, TP53INP1, TP53INP2, TRIM21, TRIM22, ULK1 (for 0010508).

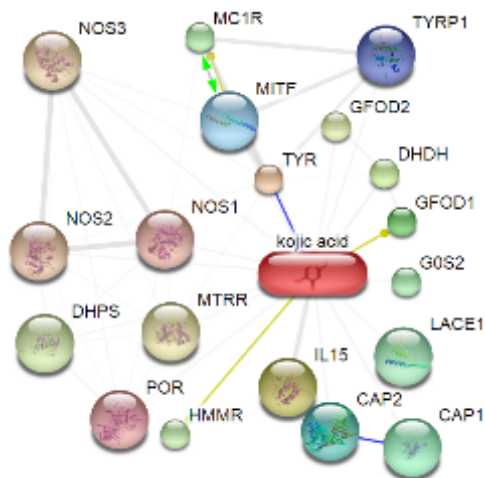


Figure 1: PPI (confidence view) of kojic acid showing nodes its proteins and edges showing its interactions. A strong bond is denoted by a thick line. Note: TYRP1: tyrosinase-related protein 1. MC1R: Melanocortin 1 receptor. MITF: Microphthalmia-associated transcription factor. TYR: tyrosine. DHDH: dihydodiol dehydrogenase. GFOD2: Glucose-Fructose Oxidoreductase Domain-Containing Protein 2. GFOD1: Glucose-Fructose Oxidoreductase Domain-Containing Protein 1. G0S2: G0 switch protein 2. LACE1: lactation elevated protein 1. IL15: Interleukin 15 receptor. CAP1: Adenylyl cyclase associated protein 1. CAP2: Adenylyl associated protein 2. HMMR: Hyaluronan mediated motility receptor. MTRR: Methionine synthase reductase. POR: Protochlorophyllide reductase. DHPS: Dihydropteroate synthase. NOS1: Nitric oxide synthase 1. NOS2: Nitric oxide synthase 2. NOS3: Nitric oxide synthase 3

Protein interaction network

The protein-protein interaction network was analyzed through Cytoscape plugin ClueGO-mediated enrichment analysis and the combined calculations were made. Thereafter, the networks were merged using advance network merge plugin to avoid duplication. The results revealed 19 target proteins in PPIN which represented 19 nodes. The nodes present in the network (Figure 2) symbolized protein targets and the edges showed interactions between them. The PPIN enrichment *p*-value was 0.74, while the clustering coefficient was 0.81. A total of 11 hubs were found on the basis of higher node degree value when compared to average node degree value of

3.16. In this case, TYR and NOS3 had the highest node degree value viz. 8. The successive proteins were NOS1, NOS2, and MTRR having the node degree value of 7 based on average node degree value (Table 1). However, the expected number of edges was 33.

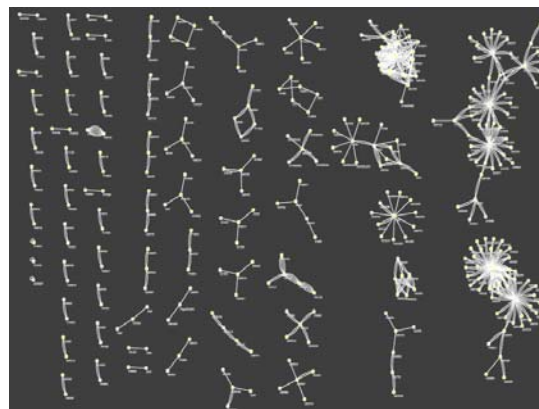


Figure 2: The protein interaction network of kojic acid

Table 1: Protein targets of kojic acid and their scores

Protein target	Score
TYRP1	4
MC1R	5
MITF	5
TYR	8
DHDH	4
GFOD2	2
GFOD1	2
G0S2	1
LACE1	1
IL15	1
CAP1	2
CAP2	2
HMMR	1
MTRR	7
POR	6
DHPS	6
NOS1	7
NOS2	7
NOS3	8

Topological analysis of network

Topological analysis revealed that there were 69 connected components of PPIN. The degree of the irregular distribution of the network gave network heterogeneity value, of 1.380. Maximum distance between vertices is called a network diameter. Its value noted in this study is 8. The network centralization value is 0.073 which is represented as the extent to which the nodes are diffused and scored centrality in a network (Table 2).

Table 2: Topological features of the kojic acid protein interaction network analyzed using Cytoscape

Topological parameter	Value
Connected components	69
Network radius	1
Network heterogeneity	1.380
Network density	0.06
Mean number of neighbors	2.216
Characteristic path length	2.875
Multi-edge node pair	249
Clustering coefficient	0.014
Network diameter	8
Network centralization	0.073
Number of nodes	342
Isolated nodes	3
Number of self-loops	4
Shortest path	6342 (5%)
Analysis time (sec)	2.785

Analyzed biological processes

Kojic acid-related target genes were analyzed using Cytoscape plugin ClueGO. This analysis resulted in a significant enrichment of 104 GO terms. These terms were further categorized into 11 subgroups (Figure 3). These groups are widely involved in several circadian regulations of gene expressions such as positive regulation of keratinocyte differentiation, protein trimerization, retinoic acid receptor signaling pathway, and positive regulation of autophagy.

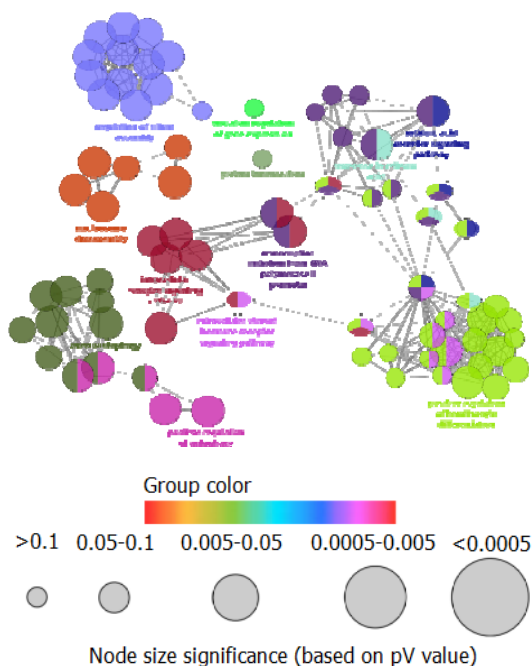


Figure 3: Functionally grouped networks, determined through ClueGO analysis to retrieve the potential targets of kojic acid. Each cluster consists of the most crucial terms only. The overlapped clusters reflect a resemblance of their functions

DISCUSSION

Kojic acid possesses antibacterial, antifungal, wound and scar healing, anti-inflammatory, and anticancer activities. The exact mechanism of action of kojic acid is still unclear, thus, system pharmacological study comprising a three-step mechanism i.e., extracting drug target, constructing the network, and analyzing pathways was designed. From results, it was found that there was a total of 19 protein targets of kojic acid and the acquired network contained 11 hubs. Most of the hubs were already reported to be involved in various diseases such as microbial infection, cancer, and aging [13,14]. These hubs are TYR, NOS3, NOS2, and NOS1. Kojic acid revealed its effect by inhibiting tyrosinase (TYR). Tyrosine (TYR) is the major hub. It is already known to be involved in the production of melanin [13]. Due to TYR insufficiency, the level of melanin pigment is raised simultaneously developing melasma, freckles, and melanosis [14]. Thus, kojic acid holds the process of converting tyrosine to melanin by inhibiting the enzyme tyrosinase. Thus, the down-regulation of tyrosinase induces the whitening effect in the skin by kojic acid and treats skin diseases [6].

There are three isoforms of NOS (NOS1, NOS2, and NOS3), which are involved in the modulation of NO (nitric oxide) synthesis in the mammalian cells. These isoforms are generally classified on the bases of their isolation and characterization [15]. The human gene that encodes eNOS is designated as NOS3 shows the highest node degree value in the present study. Kojic acid can be used as an anti-amnesic agent, since it has potential for decreasing the level of NOS in rats. Similarly, decreased melanin also exhibits amnesic effect, thus the insertion of melanin-concentrating hormone overcome this effect as MCH injection increases the NO level in the hippocampus [8]. Kojic acid is found to be associated with NOS2 in decreasing melanin pigment in the body [2]. NOS1 produces nitrogen acid (cytotoxic nitrogen oxide), which further reacts with 5,6-dihydroxyindole (DHI) and its 2-carboxylic acid (DHICA) leading to darkening of skin by producing melanin pigment. Thus, kojic acid can contribute to decrease melanin levels in the skin and prevent skin from inflammatory stimuli and oxidative tissue injury [1].

Circadian regulation of gene expression is the regulation of gene expression of the biological processes in association with time or environmental factors [16]. Many biological and physiological processes that are regulated by circadian gene expression. These are

pharmacodynamics mechanism, vision, perception of learning, physiological movement, and detoxification [3]. There are several genes such as BHLHE40, BHLHE41, and PPARA, which are linked with circadian regulation of gene expressions. GO analysis describes that the above-stated activities could be regulated by kojic acid. Another finding in this study shows that kojic acid also influences the regulation of protein trimer biosynthesis known as protein trimerization, which unites the macromolecular structures comprising three non-covalently bounded similar and dissimilar subunits. The genes linked to protein trimerization are TRIM21, TRIM22, and TRIM5. Misfolding of proteins leads to several pathological conditions such as trauma, ischemia and oxidative stress [14]; sickle cell anemia [2], decreased elasticity of red blood cells [6], and epidermolysis bullosa simplex [17]. It also plays a significant role in the production of vaccines [8].

The genes involved in positive regulation of keratinocyte differentiation are MED1, NCOA3, VDR, and TRIM16. Embryonic cells require latent quality of differentiation for organ morphogenesis, just like keratinocytes stem cells which have a high degree of differentiation rate. It could be hypothesized that positive regulation of keratinocyte differentiation is required for better morphogenesis of organs. The upregulation of keratinocyte differentiation is associated with Notch signaling [18]. It is known to be linked with processes such as transient amplifying cells and differentiated human keratinocytes [3]. Ultra-violet radiation stimulates melanocytes and releases melanin which affects the function of other keratinocytes, therefore these cells could be responsible for altered skin color. Kojic acid is known for reducing melanin pigment in cells, thus has a whitening effect on skin. Kojic acid could be linked to the downregulation of keratinocytes. In another study, it was documented that when human melanoma cells were co-cultured with keratinocytes, basal melanogenesis was inhibited [13].

The present findings also illustrate that the retinoic acid receptor (RAR) signaling pathway could also be related to kojic acid. The genes associated with the regulation of the receptor signaling pathway are *ACTN4*, *KMT2E*, *RARA*, *RARG*, *RXRA*, and *TRIM16*. The processes involved in the regulation of retinoic acid stimulus may induce some alteration in cellular activities when the genes are expressed. Various diseases related to retinoic acid receptor include cancer, embryonal carcinoma, promyelocytic leukemia

and metabolic diseases [5]. In addition, retinoic acid receptors signaling pathways are involved in ligand-controlled transcription to control various biological processes such as embryo development and organ homeostasis. Similar results were observed in the present study. Thus, it can be mentioned that the RAR signaling pathway is the main contributor in cellular functions such as differentiation, morphogenesis, and tissue homeostasis and the genetic functions such as transduction of retinoic acid signal during fetal development before birth, organogenesis, and embryogenesis [9]. Dysregulation of RAR signaling in the skin may induce inflammation and dysregulate skin barrier properties [17]. So, the importance of the RAR signaling pathway verifies the fact that kojic acid plays a significant role in RAR signaling.

Any process that triggers the rate of autophagy is known as positive regulation of autophagy. The autophagy process is up-regulated under stress, deoxygenation and other pathological conditions [9]. The dysregulation of this process results in the development of various diseases such as cancer, infections and other neurodegenerative disorder [4]. This study describes the involvement of kojic acid in the up-regulation of autophagy. Various genes involved in positive regulation of autophagy are *ATG7*, *BNIP3L*, *MEFV*, *SQSTM1*, *TBC1D5*, *TP53INP1*, *TP53INP2*, *TRIM21*, *TRIM22*, and *ULK1*. Macroautophagy could also be linked with kojic acid and is responsible for lysosomal degradation and recycling of cellular contents [1]. The genes involved in the regulation of macroautophagy are *ATG3*, *ATG4B*, *ATG7*, *BNIP3L*, *FAM134B*, *GABARAP*, *GABARAPL1*, *GABARAPL2*, *OPTN*, *SQSTM1*, *STBD1*, *TBC1D25*, *TP53INP1*, *TP53INP2*, and *ULK1*.

Figure 3 describes that kojic acid and transcription initiation from RNA polymerase 2 promoter are somehow connected to each other. Transcription initiation process assembles RNA polymerase II initiation complex at the specific region of DNA template i.e., RNA polymerase II promoter region leads to the synthesis of RNA [12]. Thus, it can be determined that kojic acid affects the synthesis of transcription factors. The genes involved in transcription initiation from RNA polymerase 2 promoter are *ESR1*, *MED1*, *MED25*, *NR0B2*, *NR1H3*, *NR1H4*, *PPARA*, *RARA*, *RARG*, *RXRA*, *VDR*, *NR113*, and *NR6A1*. Furthermore, kojic acid could also start the signaling of intracellular receptors due to the binding of hormones especially steroid hormones that induces alteration in gene expression [3].

Limitation of the study

The limitation of this *in silico* study is the use of previously discovered protein targets. Therefore, more research is still needed to investigate the rationality of these findings and perform clinical trials.

CONCLUSION

This study retrieves the protein targets as well as pathways of kojic acid. Based on the findings, kojic acid is a potential suitable remedy for treating inflammation, wounds and scar healing, uveitis, cancer and aging. Thus, network pharmacological study helps in understanding the action mechanism of kojic acid against different pathological conditions.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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