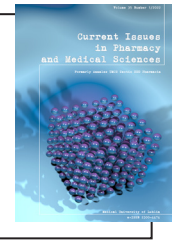
DOI: 10.12923/cipms-2025-0016 Curr. Issues Pharm. Med. Sci., Vol. 38, No.2, Pages 101-106

Current Issues in Pharmacy and Medical Sciences
Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA

journal homepage: https://czasopisma.umlub.pl/curipms



A study on design, virtual screening, and docking analysis of new ferulic acid analogs against the NF-κB therapeutic target

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ARTICLE INFO

Received 01 June 2024
Accepted 29 January 2025

Keywords:
ferulic acid,
NF-κB,
docking,
cancer,
diabetes,
inflammations.

ABSTRACT

The human transcription factor NF-κB has an essential role in inflammatory responses and carcinogenesis. Ferulic acid (FA) is one of the plant phenolic compounds that shows therapeutic potential for anticancer, anti-diabetes, anti-aging, anti-inflammatory activities, etc. This study aims to develop novel FA-based NF-κB inhibitors. The docking study of FA analogs has revealed that FA74 (-7.3 kcal/mol), FA75 (-7.2 kcal/mol), and FA71 (-7.1 kcal/mol) are top NF-κB binders, in comparison with their parental compound, FA (-4.0 kcal/mol). Accordingly, FA74 establishes four hydrogen bonds with Arg 246 (p65), Gln 606 (p50), and Ser 546 (p50); FA75 and FA71 form three hydrogen bonds with Lys 541 (p50), Arg 246 (p65), and four hydrogen bonds with Arg 246 (p65), Lys 541 (p50), Ser 546 (p50) residues, respectively. FA forms four hydrogen bonds with Arg 33 (p65), Arg 187 (p65), and Gln 606 (p50) residues. The results suggest that FA analogs (FA74, FA75 & FA 71) show promising leads that may act as effective modulators of NF-κB activity through interaction with the p50 domain or p65 Nuclear Localising Sequence (NLS) or interference of gene expression. Further MD simulations, synthesis, and pre-clinical studies may elucidate the precise relationship between NF-κB and FA analogs.

INTRODUCTION

The transcription factor, NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells), controls the gene expression of different cellular activities and inflammations [1,2]. It consists of two subunits, viz., NF-κB1 (p50) and RelA (p65), which collectively form an active form of the NF-κB dimer (Figure 1). The primary function of the canonical NF-κB pathway is to activate NF-κB in response to outside stimuli from different immune receptors [3]. In most tumors, NF-κB is mis-regulated, constitutively active and keeps cell proliferation continuous [4]. In addition, some tumor cell secretory factors induce NF-κB activation. Blocking NF-κB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents. NF-κB stimulates gene transcription for G1

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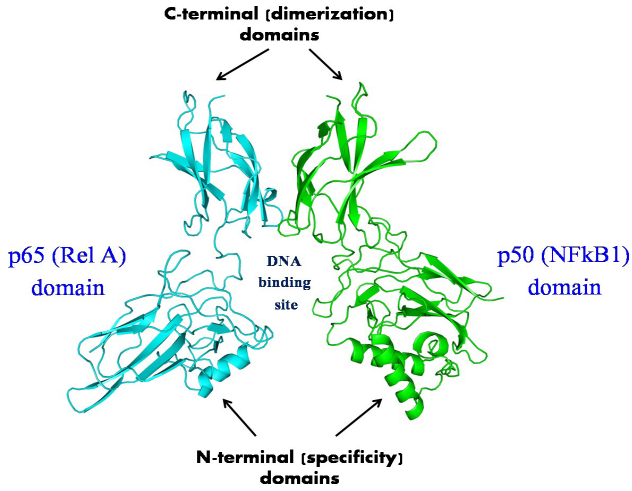


Figure 1. 3D structure of NF-κB protein

cyclins, and NF-κB-dependent cyclin D1 induction drives the activation of several target genes that block the induction of apoptosis.

Several studies have shown that mammary carcinoma cell lines and primary breast cancer cells have high or constant NF-κB DNA-binding activity [5,6]. Mutations of NF-κB are found in many cancers, although the mutation frequency of RelA and RelB is much lower than that of REL, p50, and p52 [7,8]. NF-κB exhibits its fundamental role as a transcription factor through binding with DNA as its substrate. The active site residues of NF-κB interact with dsDNA during its gene regulation mechanisms.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as Aspirin, Ibuprofen, Sulindac and Indomethacin, inhibit the activation of NF-κB in cell lines, while glucocorticoids such as dexamethasone, prednisone and methylprednisolone, are used for their anti-inflammatory properties and to prevent allograft rejection by inhibiting NF-κB. Moreover, selective estrogen receptor modulators (SERMs) such as Raloxifene and Tamoxifen used in treating osteoporosis and cancer, respectively, can potentially decrease the nuclear translocation of NF-κB in experimental Rat models [9].

Cyclosporin A, an inhibitor of B- and T-cell proliferation, by blocking the activity of calcineurin, limits NF-κB induction. Indeed, a variety of human drugs have been primarily characterized as anti-inflammatory or anti-tumor agents having NF-κB inhibitory activity. For example, auranofin is a potent inhibitor of the NF-κB pathway in response to multiple stimuli, including the proinflammatory cytokines IL-1 and TNFα and the SARS-CoV-2 infection. In contrast, auranofin stops the creation of activation complexes with NEMO when IL-1, TNF, and LPS are present [10]. Many drugs, including emetine, fluorosalan, sunitinib malate, bithionol, narasin, tribromsalan and lestaurtinib, inhibit NF-κB signaling via the regulation of IkBα phosphorylation. Others, such as ectinascidin 743, chromomycin A3, and bortezomib, utilize other mechanisms [11].

Polyphenol compounds have unique advantages in preventing cancers through their antioxidant and anti-inflammatory effects by limiting NF-κB activity [12]. They also regulate programmed cell death, migration and senescence-related signalling pathways of cancer, via the modulation of reactive oxygen species (ROS), the mitogen-activated protein kinase (MAPK) pathway, NF-κB pathways and glycolytic enzymes. Furthermore, polyphenols have been found to prevent cancer initiation (cytoprotection), progression, recurrence and metastasis to distant organs. Polyphenols hinder NF-κB expression and chromatin remodeling through modulation of epigenetically related enzymes, such as HDACs, histone acetyltransferases (HATs) and DNMTs [13-15]. Curcumin, resveratrol [16], sulforaphane [17], etc., for example, curb histone deacetylation and anti-tumor activity, while other polyphenols, such as epigallocatechin-3-gallate (ECGC) inhibit histone acetylation during epigenetic modification.

FA (4-hydroxy-3-methoxycinnamic acid) (ferulic acid), found in corn, wheat and flax, is a phenol group member [18] that exhibits therapeutic potential against a wide range of ailments, including neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunction and inflammatory diseases [19]. FA is widely used in the pharmaceutical, food and cosmetic industries. It has a protective role for the main skin structures: keratinocytes, fibroblasts, collagen and elastin. FA impedes proliferation and induced apoptosis in osteosarcoma cells through G0/G1 phase arrest and downregulated cell cycle-related protein expression, CDK 2, CDK 4, CDK 6. Moreover, FA upregulates Bax, downregulates Bcl-2, and enhances caspase-3 activity. More importantly, FA dose-dependently restricts PI3K/Akt activation [20].

FA prevents methotrexate nephrotoxicity by inhibiting the NF-κB NLRP3 inflammasome axis, inhibiting inflammasome activation, and decreasing NF-κB phosphorylation [21]. The man-made ferulic esters and ferulic amides were more effective at killing cancer cells in tests [22]. Similarly, other plant compound derivatives generated better cytotoxicity and NF-κB inhibitory activity in cancer cells [23-25].

Although FA exerts anticancer drug activity through NF-κB inhibition, it still faces obstacles such as poor solubility and weak absorption in experimental models. To overcome this problem, ferulic acid-based derivatives will be designed and tested for further biological improvements. In this study, we designed FA analogs using computational techniques and screened for their pharmacological properties, followed by a docking study against the NF-κB therapeutic target.

MATERIALS AND METHODS

Design and preparation of FA pharmacophore library

A data set of 500 FA analogs was prepared using substituents and linkers available in Chem Draw ultra 8.0 (Cambridge Soft, Cambridge, MA, USA) and Molinspiration servers [26] by inducing structural modifications onto FA. All compounds were designed using possible synthetic procedures.

Prediction of drug-likeness and ADMET of FA analogs

Druglikeness and ADMET are qualitative tools used in drug design to predict the drug properties of novel compounds before synthesis and testing. The factors of bioavailability, octanol-water partition coefficient (log P), molecular weight, polar surface area (TPSA) were analyzed using a Molinspiration server [26]. The ADMET profile included solubility, cell permeability, plasma distribution, metabolism, excretion and other toxicity parameters that were screened using the pre-ADMET server (http://preadmet.bmdrc.org/) [27,28].

Molecular docking

The crystal structure of NF-κB (PDB ID: 3GUT) with a resolution of 3.59 Å was retrieved from Protein Data Bank (PDB) (https://www.rcsb.org/). Before starting the docking, all non-protein molecules were removed from the protein file. The PRODRG server-generated FA, and its analogs [29] were pre-optimized using the MMFF94x force field.

Molecular docking was performed using the Auto Dock Vina by applying default parameters. The software tool that computes the binding energy of receptor-ligand parameters considers the stearic, hydrophobic and hydrogen bonding interactions [30]. The docking grid box size was 40×40×40 Aº,

with a grid point spacing of 0.375 Å. During the simulation, the program carried out 50 docking runs and produced multiple receptor-ligand binding conformations. The ligands were allowed to be flexible, and protein was kept rigid during docking. After each simulation, the mechanism forms a statement describing the output information for every run and group, such as binding energy (kcal/mol), dissociation constant, and other energy parameters. The lowest energy values with more clusters were selected as the best position. PyMOL v2.5.4 [31] was employed to analyze the ligand-receptor complexes.

RESULTS

We designed and evaluated a series of 500 FA analogs for their drug-likeness and ADMET profile. Of these, 160 analogs obeyed the Lipinski rule of five (TPSA (Polar surface area), molecular weight, log P, and H-bond donors/acceptors). Following this, 70 compounds (out of 160) were selected as good entities based on their ADMET profiles. The filters include plasma protein binding (extensive plasma protein binding will increase the amount of drug that must be absorbed before the unbound drug's adequate therapeutic levels are reached), MDCK cell permeability, human intestinal absorption, *in vivo* BBB penetration and *in vitro* Caco-2 cell permeability, which were predicted using the server. In this study, the selected FA analogs showed water solubility, as well as good permeability into Caco-2 cells, and the blood-brain barrier (BBB). In addition, these compounds exhibited good transport through the blood with the help of plasma proteins. Furthermore, using the pre-ADMET server, the compounds showed to be non-carcinogens and non-inhibitors to microsomal enzymes in toxicity screening.

All the selected FA analogs (70 compounds) were subjected to docking with NF-κB protein. The top three hits of FA analogs (FA74 (-7.3 kcal/mol), FA75 (-7.2 kcal/mol) & FA71 (-7.1 kcal/mol)) were captured for further analysis (Table 1). FA74 showed the highest docking energy of -7.3 kcal/mol among all. In the overall study, FA analogs revealed more docking energy than their parental compound, FA (-4.0 kcal/mol), which indicated that FA analogs are better binders to NF-κB than FA. During the experiment, binding residues of NF-κB were selected within the range of 4Å (bond length) around each compound (Figure 2).

Regarding the analogs, FA74 establishes four hydrogen bonds with Arg246 (p65), Gln606 (p50) and Ser546 (p50), and the rest of the interactions are formed by Lys218, Gln247, Phe607, Lys572, Pro543, Asn547, Lys541 and Arg354 (Figure 3A). As shown in Figure 3B & 3C, FA75 & FA71 form three hydrogen bonds with Lys541 (p50), Arg246 (p65) and four hydrogen bonds with residues Arg246 (p65), Lys541 (p50), Ser546 (p50), respectively. In contrast, the parent compound, FA, forms four hydrogen bonds with NF-κB's Arg33 (p65), Arg187 (p65) and Gln606 (p50) residues and the other interactions are formed by Lys218, Gln247, Arg246, Lys572 (Figure 3D). The hydrogen bonds formation of FA analogs with active site residues of NF-κB indicated that FA analogs are crucial binders and may act as NF-κB inhibitors.

Table 1. Details of binding energy and hydrogen bond formation by top ranked Ferulic acid analogs with NF-κB

S.No	Compound	Binding energy (Kcal/mol)	No. of hydrogen bonds	Active site residues of NFκB sharing H-bond with ligand	Other residues involved in interactions (4Å distance)
1	FA74	-7.3	4	Arg246(p65), Gln606(p50), ser546 (p50)	Lys218, Gln247, Phe607, Lys572, Pro543, Asn547, Lys541, Arg354
2	FA75	-7.2	3	Lys541(p50), Arg246(p65)	Lys218, Gln247, Lys572, Pro543, Arg354, Gln606, Phe607
3	FA71	-7.1	4	Arg246(p65), Lys541(p50), Ser546(p50)	Lys218, Gln247, Phe607, Lys572, Pro543, Asn547, Arg354
4	Ferulic acid (FA)	-4.0	4	Arg33(p65), Arg187(p65), Gln606(p50)	Lys218, Gln247, Arg246, Lys572

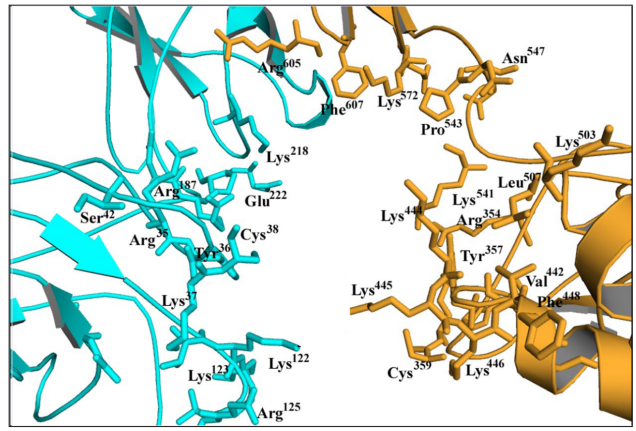


Figure 2. NF-κB active site residues

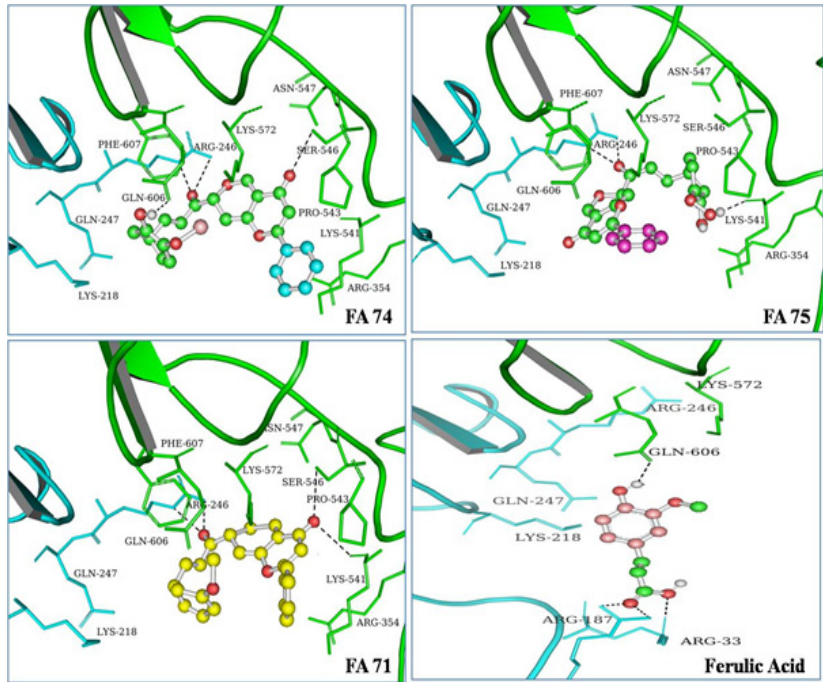


Figure 3. Protein-ligand docked complexes of a) FA74, b) FA75, c) FA71 and d) Ferulic acid (FA)

DISCUSSION

Cancer's hallmark is inflammation. This is associated with deregulating inflammatory proteins such as TNF-α, IL-1β, IL-6, NF-κB, etc. (NF-κB is one of the leading factors associated with cancer cell transformation). The FDA approves several chemotherapeutic drugs to treat various cancers and inflammatory diseases; however, they are not always highly effective for several reasons [32].

Phytocompounds possess unique chemical moieties involved in scavenging free radicals when they interfere with cellular mechanisms. These include antioxidant enzyme actions, apoptosis, cell cycle arrest, necrosis and inflammations. FA, found in the cell walls of grains, fruits and vegetables, is one of the least toxic known phytochemicals. It has an effective free radical scavenging activity, thereby exerting a wide range of pharmacological activities (among others, antioxidant [33,34], anti-inflammatory [35,36], antiviral [37], anti-fibrotic [38], anticancer [32], etc.). However, FA has certain limitations, i.e., short half-life, low bioavailability, and negligible potency. Similar analogs can be developed to overcome these limitations and further improve FA potency.

This study applied computational techniques to do so, and a set of 500 FA analogs was designed through functional group modifications. After screening through Lipinski's rule (Table 2), ADMET filters (Table 3), and docking study, three FA analogs (FA71, FA74 & FA75) were finalized as the best hits of NF-κB binders.

Table 2. Lipinski's values of selected compounds

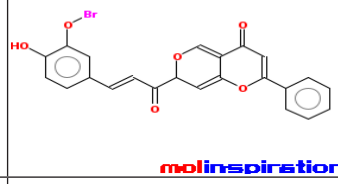
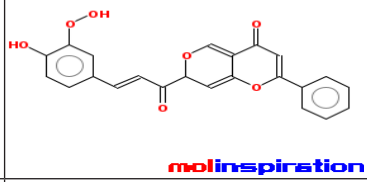
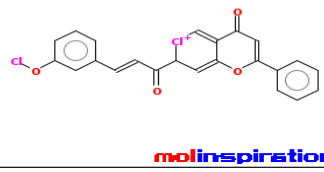
Compound	Molecular structure	Lipinski's values
FA 74		miLogP – 4.10 TPSA – 85.98 Å MW – 467.27 nON – 6 nOHNH –1 nrotb – 5 Volume – 351.09
FA 75		miLogP – 3.07 TPSA – 106.21 Å MW – 404.37 nON – 7 nOHNH – 2 nrotb – 5 Volume – 376.95
FA 71		miLogP – 2.19 TPSA – 56.52 Å MW – 426.27 nON – 6 nOHNH – 1 nrotb – 5 Volume – 351.09

Table 3. ADMET properties of selected compounds

Compound	Human Intestinal Absorption (nm/second)	*In vitro* Caco2 cell permeability (nm/second)	*In vitro* MDCK cell permeability (%)	*In vitro* plasma protein binding (C.blood/ C.brain)	*In vivo* blood brain barrier penetration
FA 74	96.64	21.21	0.03	100	0.04
FA 75	93.53	18.83	2.75	88.76	0.04
FA 71	97.44	23.11	0.1	100	0.26

Concerning the docking results, FA71, FA74, and FA75 showed themselves to be best-docked analogs of FA and were found to establish interactions with both p65 and p50 subunits of NF-κB. This indicates that these analogs were accommodated in different regions of the NF-κB active site due to their structural variation and selectivity. The variation in binding mechanisms of analogs in the interior areas of p65 and p50 domains reflects their potential as inhibitors of NF-κB.

Five members of the NF-κB/Rel family proteins viz., p50, p52, p65 (Rel-A), c-Rel, and Rel-B, are found in mammals. These form homo- or heterodimers and remain as inactive complexes with the inhibitory molecules called 'IκB proteins' in resting cells. Among these, the p65: p50 heterodimer is the most abundant form of NF-κB and is activated by pathologic stimuli via the canonical pathway. Although p50 is the predominant regulatory subunit of the NF-κB complex, the binding of FA analogs with p50 residues may modulate the NF-κB function in protein expression and different cellular processes.

Plant compounds, viz., Andrographolide [39] and Eriocalyxin B [40], as well as some marine-originated drugs, have shown anticancer activity through p50 interaction, and drugs targeted to p50 might inhibit the NF-κB signaling in various cancers [41]. The p50 domain exists in homo and hetero dimeric states in the cells. In a homodimeric state, p50 may act as an active repressor of inflammation. Since p50 over-expression is frequently observed in skin cancers, non-small cell lung carcinoma and other tumors, it is believed that the binding of FA compounds may inhibit p50 dimer's translocation or transcriptional activity [42,43]. On the other hand, FA analogs demonstrate hydrogen bonding with p65 residues, which may influence the transactivation and IkB binding property of NF-κB. Thus, the drug interaction may change the structural integrity of p65's nuclear localization sequence (NLS), thereby leading to NLS misreading by nuclear importins [44].

Docking studies of Curcumin and Tangeretin have revealed that these compounds form hydrogen bonds with active site residues of NF-κB and NLS residues of p65, thereby controlling the NF-κB function [45]. In this study, the FA analogs showed better NF-κB binders than their parental compound (FA).

The combined therapy of FA with doxorubicin has been seen to upregulate the pro-apoptotic protein expression in NF-κB-resistant cells. Its effect on the reversal of P-glycoprotein mediated drug resistance occurs via blockage of the PI3K/AKT/NF-κB signaling pathway. FA down-regulates PI3K and AKT, thereby inhibiting the phosphorylation of IkB. It also prevents the translocation of NF-κB from the cytosol to the nucleus. Previously, it has been reported that FA blocks the PI3K/Akt signaling pathway in cancer cells [46]. FA administration is also known to prevent tumor formation in oral carcinogenesis by down-regulating NFkB, COX-2 and VEGF [47].

Multiple studies have been conducted on phytocompounds, and their analogs have been tested against NF-κB in different cancer cell lines [23, 48-50]. Research indicates that a hybrid of caffeic acid-ferulic acid compound down-regulates inflammatory responses by inhibiting NF-κB

in BV2 and RAW264.7 cells [51]. Other hybrid derivatives of FA, such as ferulic acid-parthenolide (FA-PTL) and ferulic acid-micheliolide (FA-MCL) hybrid, have been evaluated as potential anti-inflammatory compounds *in vitro* [52]. It is thought that hybrid derivatives of FA enhance their potential through synergistic effects.

The ADMET profiles of FA analogs suggest that these are water soluble and more permeable to Caco-2 cells and the blood-brain barrier (BBB). Furthermore, all the title compounds exhibited more than 90% of blood absorption and plasma protein distribution (Table 3). The ADMET profiles also indicated that FA analogs have better intestinal absorption and good plasma transporting activity, suggesting that these may be better consumed from the gastrointestinal tract upon oral administration. In turn, the metabolic activity predicted that FA analogs are non-inhibitors of microsomal enzymes (CytP450), which means that the molecule will not hinder the biotransformation of its metabolized drug [53]. Moreover, the carcinogenic profile showed that all three analogs are non-carcinogenic and that not one compound of these would be bioaccumulated in the human body in long-duration treatment and would not cause cancers in the future.

The study revealed that FA analogs performed better as NF-κB binders than did their parent compound, FA. The direction of plant-based drugs research has been towards improving treatment without side effects. Hence, further molecular analysis of FA-NFkB complexes will be performed through MD simulations and in our future studies.

CONCLUSION

In summary, the in-silico findings suggest the three FA analogs, viz., FA74, FA75, and FA71, show superior binding scores with NF-κB drug targets. The hydrogen bond interactions with the active site residues of NF-κB's p65 and p50 subunits demonstrate that these compounds can effectively modulate the NF-κB activity by interfering with its gene regulation mechanisms. The binding sites of FA analogs decide the selection of their interaction mechanisms and interference mechanisms in protein-protein or protein-DNA interaction. The interior binding of FA analogs at p65 and p50 domains reflect a nature of inhibitory action against NF-κB functions. This interference could potentially down-regulate the inflammatory responses and their associated molecular targets. The pharmacokinetic and drug-likeness profiles of FA analogs are acceptable as drug candidates due to their non-carcinogenic and non-toxicity. This study could be helpful for the future design, optimization and investigation of plant-based drugs to produce more potent and selective NF-κB inhibitors.

CONFLICT OF INTEREST

All authors confirm that this article's content has no conflicts of interest.

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