*Gelidiella Acerosa*compounds Target NFKB Cascade in Lung Adenocarcinoma

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Research Article

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Abstract

In carcinogenesis, increased metabolism, abnormal functioning of mitochondria, peroxisomes, aberrant cell signaling and prolonged inflammation can result in the overproduction of reactive oxygen species (ROS). In turn, excess ROS can upregulate the expression of various signaling pathways including the MAP kinase, PI3K/Akt and NFKB cascades in cancer. The constitutive expression of NFKB causes drug resistance in lung cancer. Hence, drugs that can enhance the antioxidant activity of enzymes and regulate the NFKB activity are of prime target to manage the drug resistance and inflammation in cancer. This study evaluated the effect of compounds present in ethyl acetate extract of *Gelidiella acerosa* on inflammation and on antioxidant enzymes in lung cancer. The anti-inflammatory activity was determined under *in silicoandin vitro* conditions. The *in silico* analysis showed that the phyto-constituents of *G.acerosa*inhibit the IKBα-NFKB-p65-p50 complex in a similar way as that of doxorubicin and dexamethasone.*Similarly*,*G.acerosa*treatment enhanced the efficiency of antioxidant enzymes peroxidases and superoxide dismutase in A549 lung cancer cells. Further, the results of *in vitro* analysis showed that *G.acerosa* can inhibit the activation of NFKB, production of proinflammatory cytokines and upregulate the expression of IL 10. As inflammation causes cancer progression, the inhibition of inflammation inhibits tumorigenesis. Hence, based on the results of the study, it can be concluded that *G.acerosa* exerts anti- inflammatory activity through inhibition of NFKB cascade and moreover,the phyto-constituents of*G.acerosa* may have the potential to treat cancer successfully.

1. Introduction

Reactive Oxygen Species (ROS) are group of highly reactive molecules generated as a normal by-product of cellular metabolism. ROS are removed by the cellular antioxidant enzymes including superoxide dismutase, peroxidase and catalase families and by non–enzymes, such as the flavonoids, vitamins and glutathione to maintain homeostasis. Superoxide dismutase(SOD) are metallo enzymes, ubiquitously expressed in cells. They convert the superoxide generated to oxygen and hydrogen peroxide. The hydrogen peroxide is removed by peroxidases, thus protecting the cells from oxidative stress [1]. It is well established that increased metabolism, abnormal functioning of mitochondria, peroxisomes, cyclooxygenase, lipoxygenase, aberrant cell signaling and prolonged inflammation can result in the overproduction of ROS in carcinogenesis [2]. Furthermore, the interleukins and growth factors upregulate the production of hydrogen peroxide and nitric oxide in tumour cells [3]. The production of TNFα and IL-1β by macrophages also induce ROS generation in tumour cells. ROS upregulates the expression of various signaling pathways including the MAP kinase, PI3K/Akt and NFKB cascades in cancer [4]. In breast cancer, exposure to TNF α and IL-1β induced hydrogen peroxide production, which, in turn activated NFKB resulting in prolonged cell proliferation [5]. In the case of oral squamous cancer, an inhibition of SOD resulted in an up regulation of ROS levels which in turn activated NFKB pathway [6]. In lung cells, ROS are generated in response to environmental factors. Impaired clearance of ROS causes damage to the lung cells. The major antioxidant enzyme,glutathione peroxidase plays a vital role in detoxifying the ROS [7] and regulate the cytokine production [8]. Either the lack or loss of GPX activity

results in chronic inflammation of the lungs. Earlier studies have shown a constitutive activation of NFKB-p65 in lung cancer [9]. Moreover, the conventional therapies employed in the management of the disease also activate NFKB signaling resulting in drug resistance [10]. The activation of Rel A (p65) form of NFKB is considered as a predictive marker for drug resistance in cancer [11].

NFKB is a major transcription factor that occurs as homo or heterodimers. Among the major forms of NFKB, the Rel A – a heterodimer of p65 and p50 subunits is the predominant one. In resting cells, the NFKB dimer is bound with inhibitor (IKBα) and remains inactive. Upon activation, the NFKB translocatefrom cytosol to the nucleus and regulates the expression of nearly 200 genes involved in cell cycle, proliferation, survival, differentiation, migration, adhesion and inflammation. Activation of NFKB confers activation of these genes in cancers including lung cancer [12]. Alternatively, NFKB also suppresses the expression of tumor suppressor genes such as p53 and PTEN, thus promoting carcinogenesis. In addition, activation of NFKB-p65 induces anti-inflammation and immunosuppression in tumor induced macrophages. NFKB is a well-established transcription factor that regulates inflammation through the production of proinflammatory cytokines [13]. Hence, the regulation of NFKB directly affects tumour growth and tumour microenvironment. Thus, the inhibition of NFKB is reported to enhance the efficacy of anticancer drugs both under *in vitro* and *in vivo* conditions [14]. IL-10 is an anti-inflammatory cytokine that antagonizes the expression of TNFα by inhibiting the NFKB activity. It is a cytokine synthesis inhibitory factor reported to inhibit the production of inflammatory cytokines [15]. As the inhibition of NFKB is the primary mechanism behind the regulation of inflammatory process, the expression level of IL-10 was considered as an anti-inflammatory marker. In addition, the regulation of NFKB is a key factor in the management of inflammation and cancer [16].

Based on these reports, the drugs that can enhance the antioxidant activity of enzymes and regulate the NFKB activity are of prime target to manage the drug resistance and inflammation in cancer. Marine Natural Products (MNPs) are employed in the management of various diseases including cancer[17]. MNPs, especially terpenoids, alkaloids (Hymenialdisine and its derivatives), pigments (Prodigiosins) and steroids, macrolides, peptides, depsipeptides and polysaccharides are reported for their anti-inflammatory activities [18,19]. MNPs are reported to exhibit anti-inflammatory activity through the inhibition of NFKB.Cycloprodigiosin is a red pigment from marine bacteria which can exhibit immunosuppressive and apoptotic activities through the inhibition of NFKB [20]. The marine red algae are a rich in phytocompounds with varied bioactivities. In an earlier study, [21,22] we analyzed the anticancer and antimetastatic activities of marine red algae *Gelidiella acerosa* under both *in vitro* and *in vivo* conditions. The current study now analyzed the interaction of isolated algal compounds with IkBα-NFKB-p65-p50 complex under *in silico*, to determine the efficacy of the algal extract on SOD and POX activities in adenocarcinoma cell line A549, to analyze the expression of NFKB, proinflammatory cytokines (TNF α and IL-1β) and anti-inflammatory cytokine IL 10 under *in vitro* conditions.

2. Materials And Methods

2.1. Chemicals and reagents

DMEM, FBS, Antibiotic cocktail were purchased from HiMedia. Nitroblue tetrazolium blue, riboflavin, methionine, pyrogallol were purchased from Merck, Germany.

2.2. Seaweed collection, extraction and characterization

The seaweed was procured from the Mandapam coast of Tamil Nadu, India. It was authenticated and a voucher specimen was deposited at CMFRI, Mandapam, Tamil Nadu, India (Accession No: MMR-CMFRI17002). The seaweed was extracted as previously described [22] and the ethyl acetate extract (GAE), was investigated for anti-inflammatory activity. The structures of the algal compounds analyzedby GC-MS were retrieved from PubChem and subjected to *in silico* analysis.

2.3. Cell culture and treatment

The human adenocarcinoma cell line A549, was obtained from NCCS, Pune, India. The cells were cultured in DMEM, supplemented with 10% FBS and 1% antibiotic cocktail, incubated at 37°C, 5% CO2 in CO2 incubator. Actively dividing cells were seeded in a six well plate at a density of 1.5x105 cells/ml and incubated till they became confluent. The cells were treated with the algal extract (1.5mg/ml) for 24 hours and untreated cells represented the control. The cells were treated with lysis buffer (10mMTris pH7.5, 150mMNaCl, 0.1mM EDTA) and the cell lysate was collected, sonicated and centrifuged to isolate the total protein. The protein samples from both the control and treated cells were analyzed for their protein content by Lowry's method, and used for further analysis.

2.4. Analysis of antioxidant enzyme activity

2.4.1. Determination of SOD activity

SOD activity was determined as previously described [23]. Briefly, 300µl of the reaction mixture contained phosphate buffer (0.5M, pH7.5), EDTA (0.1mM), Methionine (13mM), Nitro blue tetrazolium (63mM), riboflavin (1.3mM) and 20 µg/ml of total cell protein. The contents were incubated for 15 minutes and the reaction was initiated by exposure to fluorescent lamp (15W) for 10 minutes. The reaction was terminated by switching off the lamp and the contents were covered with a black cloth. The absorbance was read at 560nm in Multimode plate reader. The experiment was performedin triplicate and repeated thrice and the values presented as mean ± SD (Standard Deviation).

2.4.2. Determination of Peroxidase activity

The peroxidase activity was determined based on the standard protocols [24]. Briefly, the reaction mixture contained 200µl of phosphate buffer (0.1M, pH7), 100µl of H_2O_2 (0.005M), 100µl of pyrogallol (0.01M) and 20µg/ml of total cell protein. The contents were incubated for 5 minutes at 25°C and 100µl of 2.5N sulphuric acid was added to terminate the reaction. The purpurogallin formed was measured at 420nm using a spectrophotometer. The enzyme activity was determined as the amount of protein sample that brought changes in absorbance by 0.1 minute/mg of protein. The experiment was carried outin triplicate and repeated thrice and the values represent mean ± SD (Standard Deviation).

2.5. Analysis of anti-inflammatory activity

2.5.1. Determination of anti-inflammatory activity in silico

The X-ray crystallographic structure of the receptor protein IKBα-NFKB-p65-p50 complex Homo sapiens was retrieved from the Protein data Bank (PDBID: 1NFI). The protein was prepared as per the standard protocol of SYBL X 1.3 which is an automated docking tool, designed to evaluate the interactions of small molecule inhibitors and drug molecules with various target proteins in three dimensions. The interacting residues, atoms involved, the number of polar bonds, bond lengths and energy of interaction were predicted by the docking tool. The ligands (compounds identified in GAE) were prepared as described by the ligand preparation program. The prepared ligands were docked with the receptor protein and the interaction was visualized through Pymol. The interaction with anticancer drug, doxorubicin and anti-inflammatory drug, dexamethasone with NFKB were evaluated and the results of the interaction were taken as reference standards.

2.5.2. Determination of anti-inflammatory activityunder*in vitro*condition

The efficacy of GAE on inflammation was determined by analyzing the expression levels of NFKB-p65, proinflammatory cytokines TNFα and IL1β by Real- Time PCR. Total RNA was isolated from the GAE treated A549 cells by TRIzol method, followed by synthesis of cDNA using the high capacity cDNA Reverse Transcription kit (Applied Biosystem). The cDNA was utilized for analysis of gene expression using BioRad SYBR Green PCR Master mix in CFX96 TOUCH BioRad. The program (95°C for 3 minutes, 40 cycles of 95°C for 15 seconds and 55°C for 30 seconds) was followed. Melt curve analysis was also carried out. The primers used are NFKB-p65 (F) 5'-ATCCCATCTTTGACAATCGTGC-3', (R) 3'-CTGGTCCCGTGAAATACACCTC-5', TNF α (F) 5'-CCCAGGGACCTCTCTCTAATCA-3', (R) 3'-GCTTGAGGGTTTGCTACAACATG-5', IL 1β (F) 5'-AAATACCTGTGGCCTTGGGC-3', (R) 3'-TTTGGGATCTACACTCTCCAGCT-5', IL 10 (F) 5'-CATCGATTTCTTCCCTGTGAA-3', (R) 3'-TCTTGGAGCTTATTAAAGGCATTC-5', β Actin (F) 5'- TAGAAGCCTCTTCATGGACAAC-3', (R) 3'-GTATCAGGCATGCAACACAAG-5'. The analysis was carried out in triplicate and the fold change in gene expression was determined based on $2^{-\Delta\Delta ct}$.

2.6. Statistical analysis of data

All the control and test data were analyzed by using Student's t- test and ANOVA. All values are presented as mean ± Standard Deviation (SD). Test and control data were compared statistically and a value of $p<0.05$ was taken as significant.

3. Results

3.1. *Algal compounds enhanced SOD and POX activity*

The SOD and POX activities of the A549 cells treatedwith or without GAE were determined. The results showed that, sample from treated cells exhibited strong SOD activity and removed the superoxide anions generated more efficiently than the untreated sample. Similarly, the level of peroxidase activity was increased in the GAE treated cells when compared to the control cells. The protein lysate from GAE treated A549 cells scavenged the H2O2 more effectively than the protein lysate from untreated cells. The results are shown in **Fig.1**. The antioxidant efficacy of GAE was previously determined by DPPH assay [21] and the outcomes of the current study strongly correlated with our previous observation.This is due to the increased activity of superoxide dismutase and peroxidases which are generally down regulated or inhibited in cancer. The results strongly confirmed the antioxidant efficacy of the algal compounds to detoxify the ROS.

3.2. *Algal phytocompounds interact with NFKB*

IKBα-NFKB-p65-p50-complex is made up of 6 chains such as, chains A&C-p65 subunit, chains B&D- p50 subunits and chains E&F- IKBα. Chain A constitutes 301 residues, chain B has 107 residues and chain E has 213 residues. They are represented by 3 sequence-unique entities (**Fig.2**).In this study, the interaction of IKBα-NFKB-p65-p50-complex with the anticancer drug doxorubicin and the anti-inflammatory drug dexamethasone (**Fig.3**) was performed. The results showed that, doxorubicin interacted with residues GLN 162, ARG73, ASN138, ASN 139 and ARG 174 of NFKB complex. The interaction was stabilized by 8 polar bonds with bond length varying between 1- 3Å.Similarly, dexamethasone was found to interact with GLU 92, ARG174, 95 & 73. The interaction was stabilized by 5 polar bonds with bond lengths between 1-3 Å. The results of *in silico* analysis are shown in **Fig.3**and Table **1**.

The structures of the algal compounds identified by GC-MS were downloaded from PubChem and docked with IKBα-NFKB-p65-p50 complex using the SYBL X 1.3 docking suite. The results showed that, among the 15 algal compounds identified by GC-MS, only 6 compounds namely n-heneicosylformate, n-hexadecanoic acid methyl ester, 1, 2 benzenedicarboxylic acid mono ester, 6,4,10 trimethylpentadecanone and Carbamic acid phenyl (2 nitro phenyl) methyl ester interacted with the NFKB complex (**Fig.4**).The total score and C scores of these interactions were calculated based on the energy required for binding and number of bonds involved. The resultsare shown in Table **2**.

The outcomes of the *in silico* analysis showed that, both the standard drugs doxorubicin and dexamethasone interacted with the target protein at some common residues (ARG 174, ARG 95 &ARG 73). Hence, these residues are essential for regulation of NFKB activity in both cancer and inflammation. Similarly, the algal compounds also interacted with residues ARG 174 &ARG 95 of NFKB complex. The results further showed that, the algal compounds interacted and regulated the inhibition of NFKB complex in a way similar to the standard drugs.

3.3. *Algal compounds inhibited NFKB*

The activation of NFKB is essential for mediating the secretion of cytokines and hence, inflammation. Therefore, to evaluate the involvement of NFKB in the inflammatory response, this study analyzed the

expression levels of NFKB in GAE treated and control A549 cells. The experimental data are presented in **Fig.5.** The outcomes of the Real – Time PCR analysis showed that the expression of NFKB was downregulated in GAE treated A549 cells when compared to untreated control cells.The treatment with GAE significantly ($p<0.05$) suppressed the expression levels of NFKBp65 in A549 cells. These findings suggest that GAE can suppress inflammation. As the activation of NFKB is essential for the production of proinflammatory cytokines, the expression levels of TNFα and IL 1β in the GAE treated and control cells were also investigated for comparison. The results showed that GAE treatment significantly ($p<0.05$) decreased the expression levels of both TNFα and IL 1β cytokines. These outcomes demonstrated that GAE can down regulate the secretion of proinflammatory cytokines through the inhibition of NFKB phosphorylation.

3.4. *Algal compounds up regulated IL-10*

As GAE treatment inhibited the expression of NFKB-p65 and proinflammatory cytokines, the study further investigated the efficacy of GAE on the expression of the anti-inflammatory marker IL-10. Also earlier studies have shown that the deficiency of IL 10 induced inflammation and that the overexpression of IL 10 caused tumor rejection *in vivo.* The outcomes of the current study showed that the expression of IL-10, the major anti-inflammatory cytokine transcribed by NFKB, was decreased in the control cells whereas the expression levels of IL 10 was upregulated in the GAE treated cells (**Fig.5**). These results revealed that IL 10 is probably exerting anti-inflammatory activity by inhibiting the production of TNFα and IL 1β, through the inhibition of NFKB activation. These findings strongly support the results of previous reports where IL 10 is shown to suppress the secretion of inflammatory cytokines especially IL 1β, IL 6, IL 8 and TNF α by preventing gene transcription of NFKB to the nucleus [25].

4. Discussion

NFKB is the most frequently activated pathway in chronic inflammation and also in lung cancer [26]. Since the inhibition of NFKB can enhance the sensitivity to anticancer drugs, research is focused to eventually discover potent NFKB inhibitor(s) for lung cancer chemoprevention. Chemoprevention requires the continued usage of preventive drugs which may result in the intolerable side effects [27].

Hence, natural products, dietary components of vegetable origin, crude extracts of medicinal plants and fruits are more preferred than their synthetic counterparts in lung cancer prevention. Based on these reports, the current study was designed to analyze the anti-inflammatory efficacy of GAE both under *in silico* and *in vitro*conditions.

The outcomes of the study revealed the efficacy of GAE to enhance the activity of the antioxidant enzymes superoxide dismutase and peroxidase, thereby protecting against ROS-induced cellular damages and ROS-induced activation of signaling pathways including the NFKB in A549 cells. The outcomes of the current study are in line with earlier studies where *G.acerosa*was shown to regulate SOD activity in Alzheimer's disease [28] and to protect human peripheral mononuclear cells from TDCC-induced toxicity [29]. As excess ROS are associated with cellular damage, inflammation and cancer

progression, the enhanced activity of antioxidant enzymes may confer protection against free radical damage to cells [30].

Furthermore, the results of *in silico* analysis in this study revealed that the compounds in GAE targeted specific amino acidsasdoxorubicin and dexamethasone. In addition, the compounds in GAE interacted with the regulatory residues of NFKB which can modify the activation of NFKB. These data corroborate with earlier *in silico* studies where standard anti-cancer and anti-inflammatory drugs were reported to interact with NFKB [31].

The findings of the *in vitro* analysis showed that GAE treatment prevented the activation of NFKB and thereby inhibited the production of proinflammatory cytokines which mediate the inflammatory response in cancer. These data correlated with the earlier reports where algal extracts from *D.salina* were shown to decrease the production of inflammatory cytokines through the inhibition of NFKB cascade [32, 33]. Similarly, plant extracts are also shown to inhibit cytokine secretion through the inhibition of NFKB [34]. Furthermore, GAE treatment also increased the expression of anti-inflammatory cytokine (IL 10), thereby revealing the mechanism by which GAE can prevent inflammation in cancer. The current data correlated closely with previous findings [33] which revealed similar regulation of inflammation by IL 10. The results of the current study strongly support the anti-inflammatory activity of GAE since it can inhibit both NFKB and pro-inflammatory cytokines.

Together, the *in silico*and*in vitro* results show the capacity of the algal compounds to regulate NFKB-p65, thereby, offering protection from inflammatory cytokines. Furthermore, the upregulation of anti-inflammatory marker confirms the anti-inflammatory potential of the algal compounds. Our previous studies [21, 22] has revealed the anticancer and antimetastatic properties of the algal extract and this current investigation has revealed the anti-inflammatory property of the extract both under*in silico*and *in vitro* conditions. As inflammation can cause cancer progression, then the inhibition of inflammation must in turn inhibit tumorigenesis.

5. Conclusion

Based on the outcomes of the study, it can be concluded that the compounds in GAE seems to exert its anti- inflammatory property through the up-regulation of IL 10 expression, thereby inhibiting NFKB activation and subsequently the production of inflammatory cytokines in lung cancer. GAE compounds are effective as the standard drugs such as doxorubicin and dexamethasoneand target the same aminoacids to regulate NFKB and *invitro* analysis also supported the same. Overall, our results suggest that GAE compounds have the potential to remove ROS and thus can control various diseases mediated by oxidative stress. However, further studies are warranted.

Abbreviations

GAE *Gelidiella acerosa* ethyl acetate extract

NFKB Nuclear Factor Kappa B

TNF α Tumour Necrosis Factor α

IL 1β Interleukin 1β

IL 10 Interleukin 10

ROS Reactive Oxygen Species

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The datasets used and analyzed during the current study are available fromthe corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

SMF performed the experiment, collected and analyzed the data, SH designed and conceived the study.Both the authors wrote and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable.

Consent to participate

All the authors agreed to participate in the scientific work.

Consent for publication

All the authors agreed to submit the manuscript.

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Tables

Table 1. Molecular docking analysis of standard drugs with NFKB-p65.

Compound	Interacting residues	Bond length Å	Total score	C score
Doxorubicin	GLN 162	1.3	7	4
	ARG 73	1.8		
	ASN 138	1.4		
	ASN 139	2.6		
	ARG 174	2.0		
Dexamethasone	GLN 922	1.6	7	5
	ARG 95	1.5		
	ARG 73	1.4		
	ARG 174	2.3		

Table 2 Molecular docking of GAE compounds with NFKB-p65

Compound	Interacting residues	Bond length Å	Total score	C score
Heneicosylformate	ARG 174	2.2	7	5
n-hexadecanoic acid	THR 164 ARG 95	1.9 2.3	7	3
Hexadecanoic acid methyl ester	ARG 174	2.0	6	4
Mono(2 ethyl-6-(tetrahydropyranoxy)hexyl)Pthalate	ARG 96	1.6	5	3
6,10,14-trimethyl Pentadecan-2-one	ARG 96	1.4	5	4
Methyl(((2-nitro-4- (phenylmethoxy)] thioxomethyl)carbamate	ARG 95	1.7	3	3

Figures

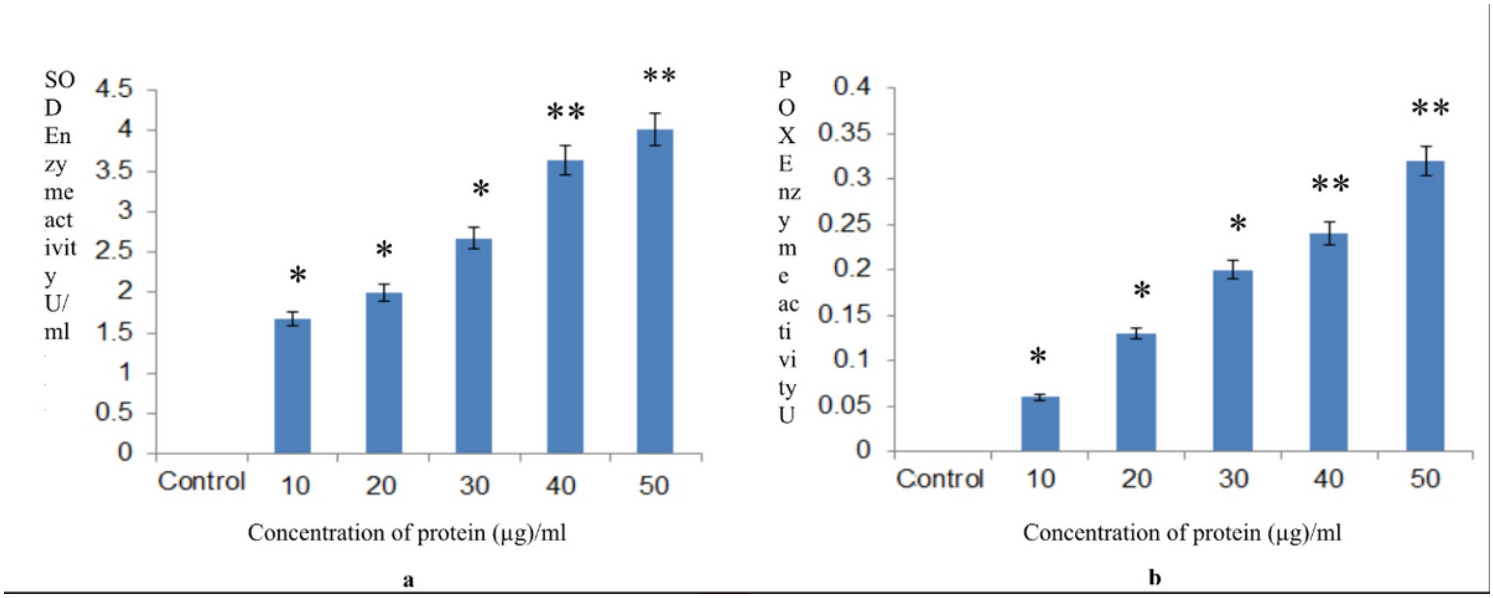

Figure 1

Analysis of antioxidant activity in GAE treated cells Effect of GAE on (a) SOD, (b) POX activities in A549 cells. Values are presented as mean ±SD from three independent experiments conducted in triplicates.*p < 0.05 for different values compared to control

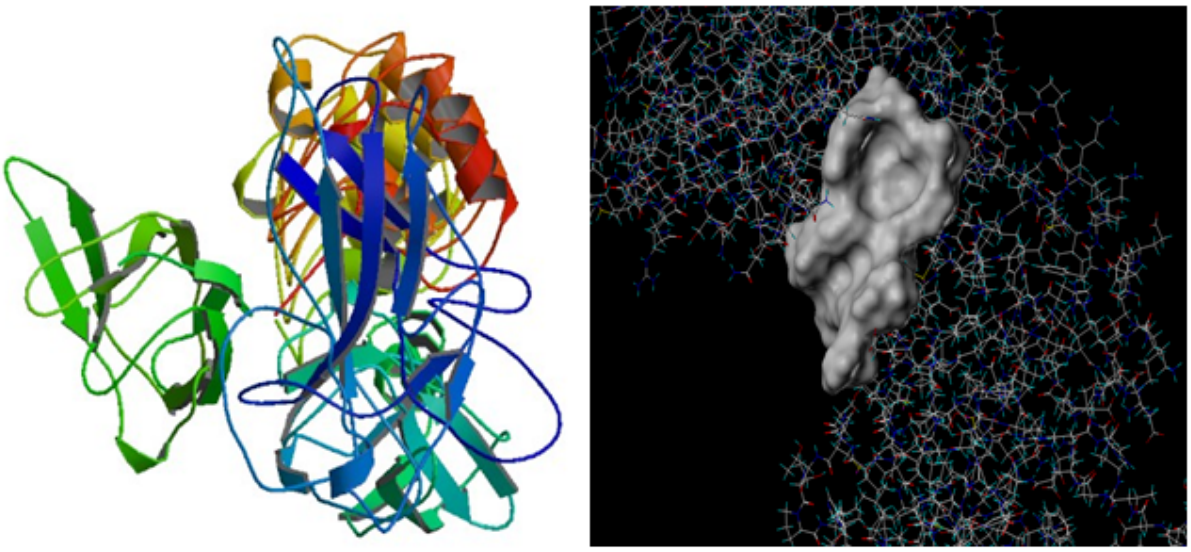

Figure 2

(a) X ray crystallographic structure of IKBα- NFKβ-p65\p50 complex (b) Pocket of IKBα-NFKB-p65\p50

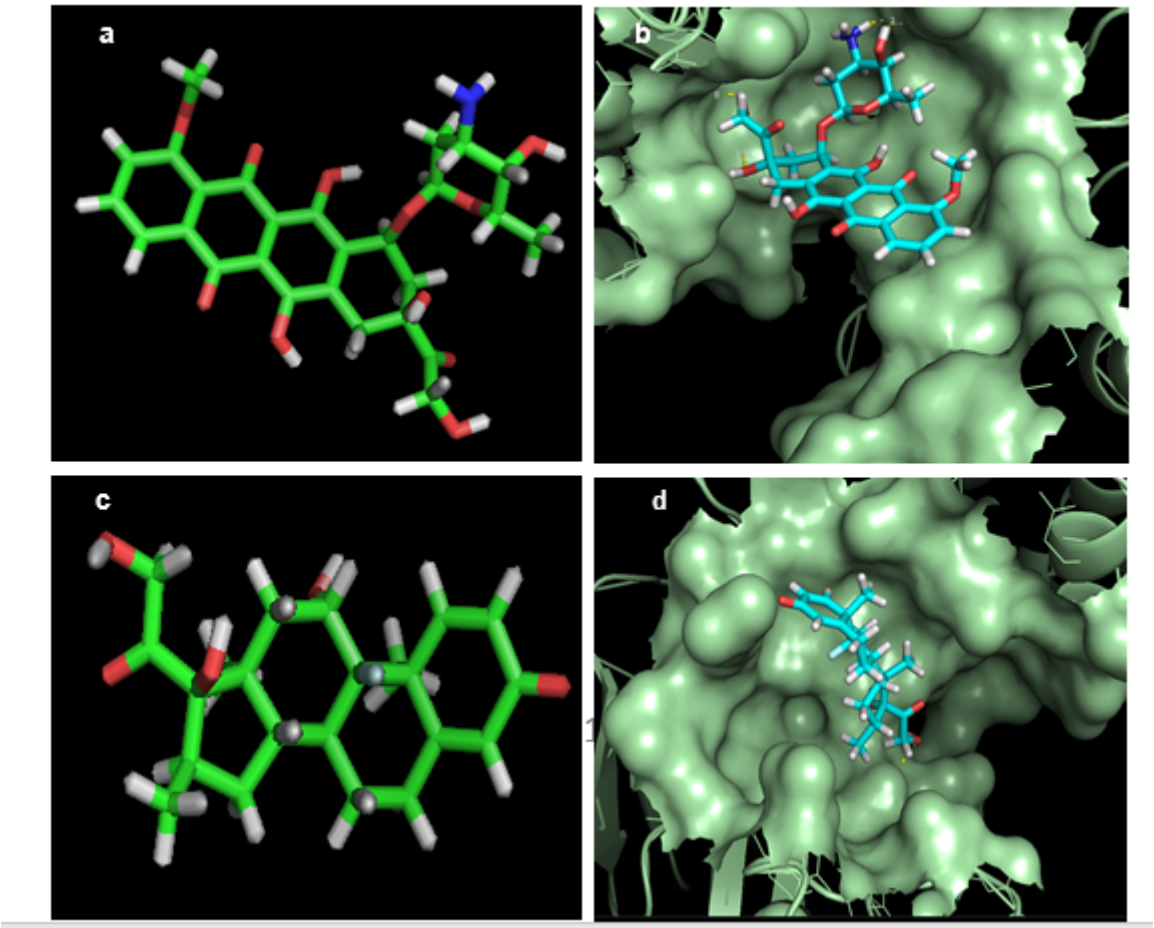

Figure 3

Molecular interaction of IKBα-NFKBp65\p50 with standard drugs(a) Structure of Doxorubicin (b) Interaction of doxorubicin with IKBα-NFKBp65\p50 (c) Structure of Dexamethasone (d) Interaction of IKBα-NFKBp65\p50 with Dexamethasone.

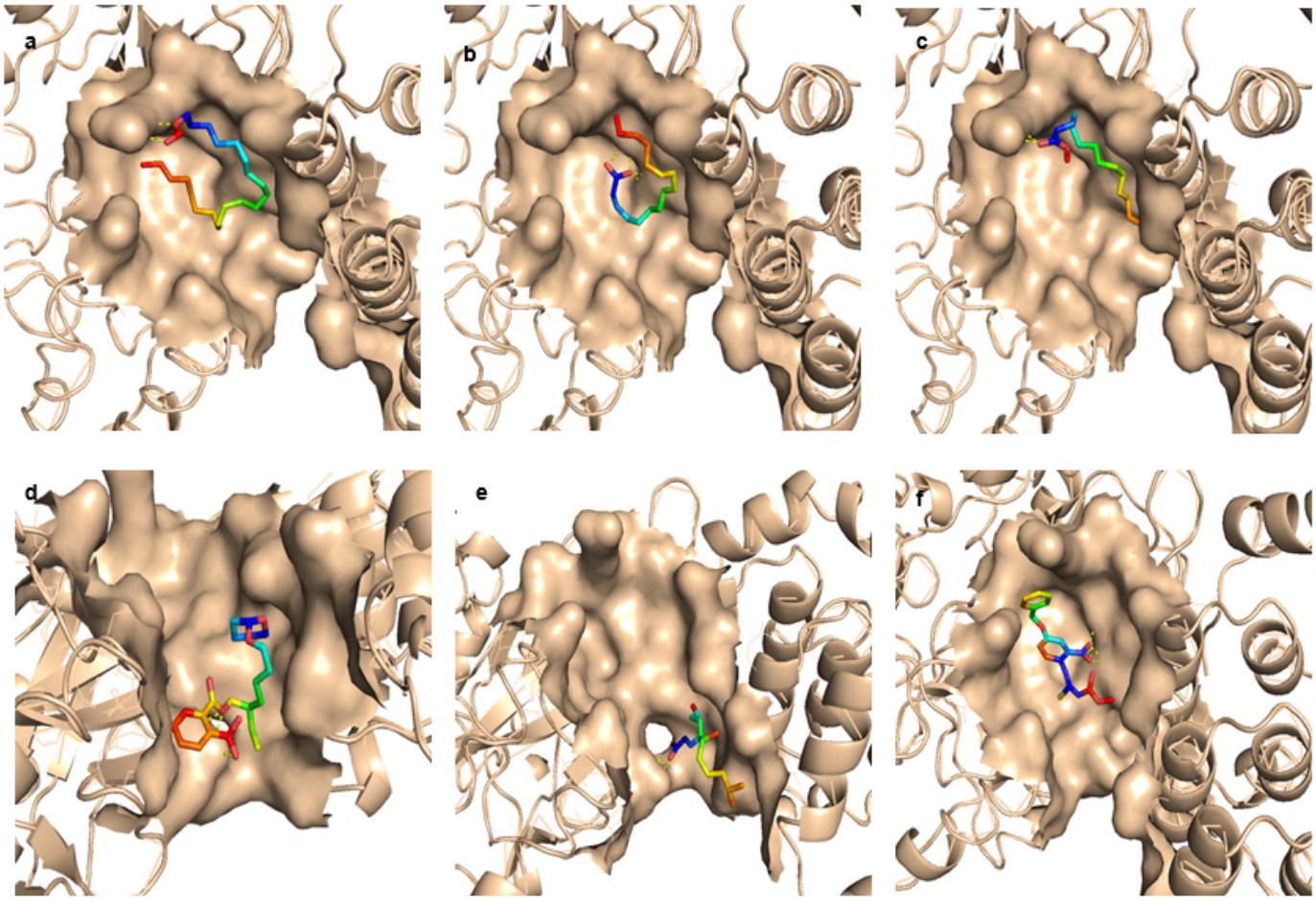

Fig.4

Figure 4

Molecular interaction of IKBα-NFKB-p65/p50 with GAE compounds(a)Heneicosylformate(b) n-hexadecanoic acid (c)hexadecanoic acid methyl ester (d) Mono (2 ethyl-6-(tetrahydropyranoxy)hexyl)Pthalate(e) 6,10,14-trimethyl Pentadecan-2-one (f) Methyl(((2-nitro-4-(phenylmethoxy) phenyl)amino) thioxomethyl)carbamate.

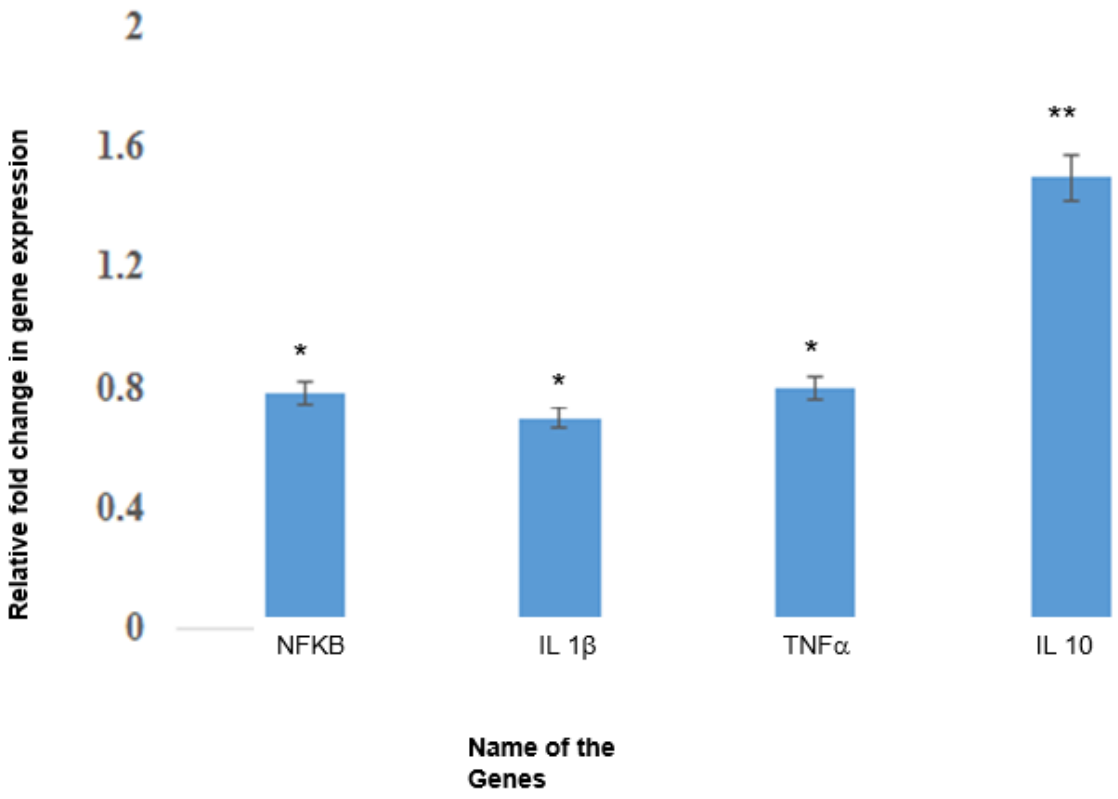

Fig.5

Figure 5

Analysis of anti-inflammatory activity of GAE in vitro(a) TheReal - Time PCR data showed the down-regulation of NFKB-p65, IL 1β and TNFα expression accompanied by an up-regulation of IL 10 expression in GAE treated cells.The expression levels of pro-inflammatory cytokines (TNFα,IL-1β) and the activated form of NFKB-p65 was decreased in GAE treated cells. The expression of anti-inflammatory cytokine (IL-10) was upregulated in treated cells when compared to the control cells. β actin was used as control. Values represent mean ± SD. *$p < 0.05$, **$p < 0.01$ compared to control. n =3.