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Original Article

Phytochemical Analysis of *Cinnamomum zeylanicum* Bark and Molecular Docking of Procyanidin B_2 against the Transcription Factor Nf- κB

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ABSTRACT

Introduction: Identification of novel natural antioxidant compounds is a highly demanding avenue of therapy for oxidative stress induced diseases. The bark of *Cinnamomum zeylanicum* commonly known as Ceylon cinnamon is commonly used in Ayurvedic medicine. **Method:** In this study, the methanolic extract of bark was subjected to GC-MS, UV absorption and TLC techniques to analyze the presence and to elucidate the structure of proanthocyanidins present in the bark. In the latter part of the study, chemdraw structure of the identified procyanidin B_2 was subjected to *in silico* molecular drug docking analysis using GOLD to find out its inhibiting efficacy against NF-κB (1NFI). **Results**: The phytochemical analysis supported the presence of a proanthocyanidin compound, procyanidin B_2. The constitutive or aberrant activation of the transcription factor, NF-κB pathway is often noticed in many cancers, autoimmune disorders, pulmonary, cardiovascular, neurodegenerative and skin diseases. The docking of procyanidin B_2 with NF-κB revealed its inhibiting efficacy by binding to active site of NF-κB and thus could inhibited the nuclear translocation and DNA binding of p50/p65 heterodimer to κB DNA sequences. **Conclusions:** Thus, procyanidin B_2 can act as the inhibitor for NF-κB. So, procyanidin B_2 present in *C. zeylanicum* bark can be used as a potential lead compound for drug development against cancer and other oxidative stress disorders.

Key words: *Cinnamomum zeylanicum*, Gold, Molecular docking, NF-κB, Procyanidin B_2.

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INTRODUCTION

Recently antioxidants have attained very important role because of their potential as therapeutic and prophylactic agents in various diseases. Traditional knowledge about medicinal plants has continuously direct the search for new cures. Supplementation of herbal antioxidants is necessary to suppress the oxidative stress in a healthier way. Use of man-made antioxidants like butylated hydroxy toluene (BHT) and butylated hydroxyl anisole (BHA) are restricted due to their side effects.[1] Proanthocyanidins are flavonoids widely distributed in plants. Proanthocyanidins better known as condensed tannins are oligomeric and polymeric flavan-3-ols.[2] Many plants produce proanthocyanidins in their fruits, bark, leaves and seeds. Proanthocyanidins are of great interest in nutrition and medicine because of its antioxidant, anti-allergic, anticancer, blood pressure and cholesterol lowering effects.[3] Over the past years proanthocyanidins supplements have become popular in the form of oligomeric proanthocyanidin complexes (OPCs) for example grape seed extracts and maritime pine bark extracts.[4]

Cinnamomum zeylanicum bark (family: Lauraceae) is commonly called as cinnamon. *C. zeylanicum* is a small and evergreen tree, most well-known for its bark. Cinnamon is extensively used in commercially available products for its culinary value and it is almost entirely obtained from cultivated plants.[5] It has been used as a spice and flavouring agent. A variety of pharmacological effects have also been observed with its use. The literature survey shows that bark of *C. zeylanicum* contains copious amounts of phenolic compounds which inhibit peroxidation reaction and therefore can be expected to prevent various chronic illnesses.[6] So, in this study, methanolic extract of *C.zeylanicum* bark was analyzed for the presence of proanthocyanidins (polyphenolic compound).

The NF-κB family consists of a group of eukaryotic inducible transcription factors.[7] NF-κB regulates the expression of genes that regulate inflammatory response, transformation, tumor promotion, tumor invasion, angiogenesis and metastasis. Activation of NF-κB is a tightly regulated event. In normal cells, NF-κB becomes activated only after the appropriate stimulation and then up regulates the transcription of its target genes. Thus, NF-κB activation is an inducible but transient event in normal cells.[8] NF-κB family comprises of many homo- and heterodimers. A commonly known NF-κB consists of p50/p65 heterodimer. It is primarily composed of proteins with molecular masses of 50 KD (p50) and 65KD (p65) and is retained in the cytoplasm by its inhibitory subunit, IκBα.[9] In response to a variety of stimuli including physical and chemical stresses, cytokines, reactive oxygen intermediates and ultraviolet light, the latent cytoplasmic NF-κB/IκBα complex is activated by the I-κB Kinase (IKK) complex.[10] IKK is formed by three distinct subunits: IKKα, IKKβ and IKKγ. The activation of IKK complex leads to the phosphorylation by IKKβ of which targets IκB for ubiquitination and degradation by the 26S proteasome. The unmasked NF-κB can then enter the nucleus and binds to the DNA target elements present in the promoters of NF-κB regulated genes, as well as to co-activators of gene transcription to activate target gene expression.[11] Deregulation and constitutive or aberrant activation of the NF-κB pathway has been observed in and attributed to the development of a variety of human ailments including cancer.[12]

Hence, search of natural *C.zeylanicum* bark derived proanthocyanidin which can inhibit NF-κB is the objective of this study.

MATERIAL AND METHODS

Collection of plant material

The bark of *C. zeylanicum* was collected from Anjarakandy Cinnamon Estate, Kannur district, Kerala and the methanolic extract was prepared.

Phytochemical Analysis

The methanolic extract of *C. zeylanicum* bark was subjected to UV absorption in the range 190 – 400nm in a UV-visible spectrophotometer. Methanolic extract of *C. zeylanicum* bark was subjected to Reverse phase HPLC (Shimadzu chromatograph) analysis, a 250 mm reverse phase C-18 ODS column with UV visible detector was used. Methanolic extract of *C. zeylanicum* bark was dissolved in appropriate volume of HPLC grade methanol and 20µl was injected into the apparatus and detected at 280 nm.

Column and thin layer chromatographic separation and detection of proanthocyanidin rich fraction

The results of UV and HPLC analysis of methanolic extract of *C. zeylanicum* bark revealed that the bark is rich in flavonoids and phenolic compounds. Hence, an attempt was made to separate phenolics rich fraction from methanolic extract of *C. zeylanicum* bark using column chromatography.

25 grams of methanolic extract of *C. zeylanicum* was dissolved in 25mL of methanol and applied onto chromatography column (2.5×30 cm) packed with silica gel equilibrated with methanol. The column was developed using gradient solvent systems with mobile phase (eluent) of increasing polarity as follows:

Hexane (100%), hexane:chloroform (80%:20%), hexane:chloroform (70%:30%),hexane:chloroform 50%:50%, hexane: chloroform (30%:70%), chloroform (100%), chloroform: ethyl acetate (90%:10%), chloroform: ethylacetate(70%:30%) chloroform:ethylacetate (50%:50%), chloroform: ethylacetate (30%:70%), ethyl acetate 100%, ethyl acetate:ethanol (70%:30%), and ethyl acetate:ethanol (50%:50%). The fractions (7ml) were collected using a fraction collector. All the fractions were then successively concentrated in a rota evaporator. The concentrates were analysed for the presence of proanthocyanidins by Thin Layer Chromatography (TLC).[13]

Precoated TLC plates with silica of 100x200 meshes (Merck) were used for the analysis. The collected fractions were spotted on TLC plates and the plates were developed with 3 different solvent systems - Hexane, chloroform and toluene & ethyl acetate (1:1). After the run the plates were analyzed for the presence of coloured spots under UV light. The TLC plates which gave spots under UV light were then dipped in vanillin-HCl (10% vanillin in ethanol: concentrated HCl in 2:1 ratio) and heated to see the red spots in day light.[14] Those fractions which gave similar spots and Rf value as standard procyanidin B_2 were pooled together to get procyanidin B_2rich fraction. The resultant procyanidin B_2 rich fraction was subjected to GC-MS (Shimadzu) using procyanidin B_2 as standard.

Molecular docking of procyanidin B_2 against NF-κB

The latest version of Gold 3.0.1 program was used for molecular docking of procyanidin B_2 against NF-κB.

Protein preparation

The 3D structure of the target protein NF-κB (PDB id: 1NFI.pdb) was retrieved from Protein Data Bank (PDB). It is a complex of p50-p65 hetero dimer bound to IκBα inhibitory protein. p65 subunit contains 301 residues, p50 with 107 residues and IκBα with 213 residues (Figure 1A).

The first step in preparation of the target protein 1NFI.pdb was to remove all waters as well as other non-protein molecules. For this purpose, Deep view (Swiss-PDB viewer)[15] was used. The inhibitor IκBα was then removed and the resulting *.pdb file was extracted and imported into MOE (Molecular Operation Environment, 2005). The "wash" function in MOE[16] was used to add explicit hydrogen atoms and to set atom ionization states based on their formal charges. Finally, the protein structure was exported as *.pdb file for input into GOLD.[17] The resulting *.pdb file was examined in order to identify a suitable binding site center atom for purpose of defining the binding site in the docking software, GOLD. The nitrogen atom in K 221 of p65 was selected.[18] This residue is located deep in the pocket close to the center of the bound IκBα.

Ligand preparation

The structure of procyanidin B_2 (Figure 1 B) was drawn using Chemdraw[19] and exported to MOE as a *.mol file. Hydrogen atoms were added to the two dimensional structure and atom ionization set to formal charge using the "wash" function in MOE before conversion to three-dimensional structure. The structure was then exported in *.sdf format for input to GOLD.

Molecular docking

The 3D structure of procyanidin B_2 was docked into the binding site of prepared target protein, 1NFI. Docking experiments were performed using the default GOLD fitness function (VDW = 4.0, H-bonding = 2.5) and evolutionary parameters: population size = 100; selection pressure = 1.1; operations = 100,000; islands = 5; niche size = 2; migration = 10; mutation = 95; crossover = 95. The number of dockings to be performed on ligand was specified under GA runs: by default this value is 10.[17] The nitrogen atom on K221 was selected as the binding site center for all calculations.

Ten docking runs were performed. If at any time 3 of the 10 poses were within 1.5 A° rmsd of each other, the docking run was terminated. All poses and the corresponding Gold scores were outputted into a single *.sdf file and text file respectively. The lowest-energy poses obtained from this stage were selected and were then rescored using the Gold Score function. Files containing best fitness function were saved.

RESULTS AND DISCUSSION

Figure 2 A shows the UV absorption spectrum of the methanolic extract of *C. zeylanicum* bark which revealed a peak at 220 nm, a small peak at 278 nm and a sharp peak at 300 nm. The peak at 278 nm in UV spectrum supports the presence of phenolic compound having absorbance at 278 nm in the bark extract.

The RP-HPLC spectrum at 280 nm of methanolic extract of *C. zeylanicum* bark is shown in Figure 2 B. Seven major peaks were observed.

Proestos *et al.*[20] identified and quantified the phenolic compounds in *Vitex agnus-castus, Origanum dictamnus, Teucrium polium, Lavandula vera* and *Lippia triphylla* by RP-HPLC monitored at 280 nm and GC-MS by comparing them with spectrum of standards and reported the presence of caffeic acid (0.12-0.93 mg 100 g^{-1}), ferulic acid (0.34-1.52 mg 100 g^{-1})and (+)-catechin (0.22-0.43 mg 100 g^{-1}) in the dry samples of the selected plants. Basu and Maier[21] studied the *in vitro* antioxidant activities of berry fruits and reported a positive correlations between IC_{50}values for different radical scavenging activities and different polyphenolics. Red raspberry extract had the highest Pearson's coefficient values (0.952–1) between total phenolics, flavonoids, and proanthocyanidins and DPPH and superoxide radical scavenging activities. Prabha

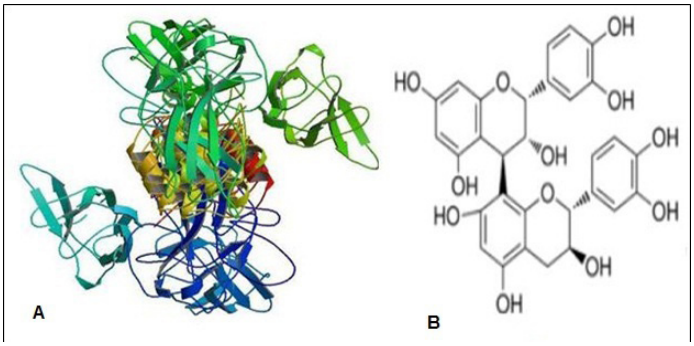

Figure 1A: Target protein NF-κB (1NFI); B. Structure of procyanidin B_2

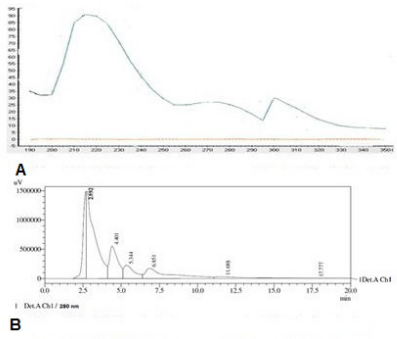

Figure 2A: UV absorption spectrum of methanolic extract of *C. zeylanicum* bark B: HPLC analysis of methanolic extract of *C. zeylanicum* bark.

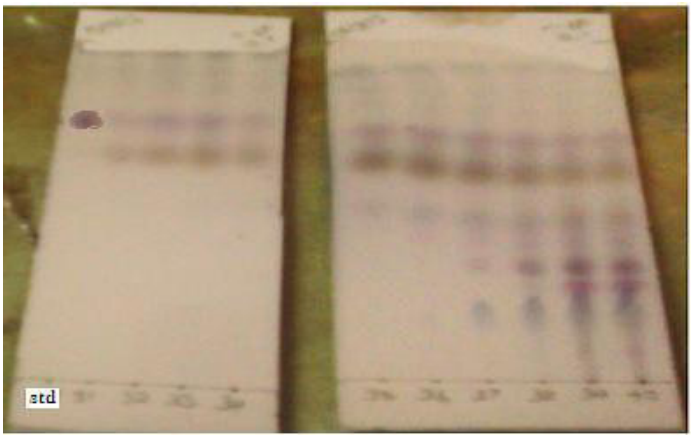

Figure 3: TLC plates dipped in Vanillin HCl.

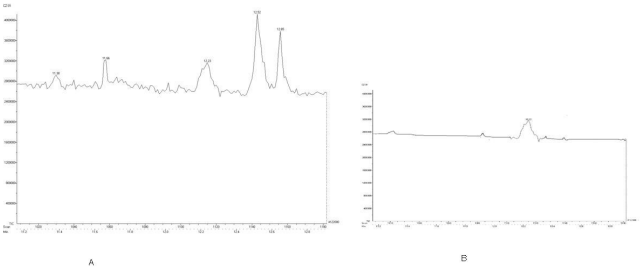

Figure 4A: GC-MS analysis of proanthocyanidin rich fraction; B: GC-MS analysis of standard (procyanidin B_2).

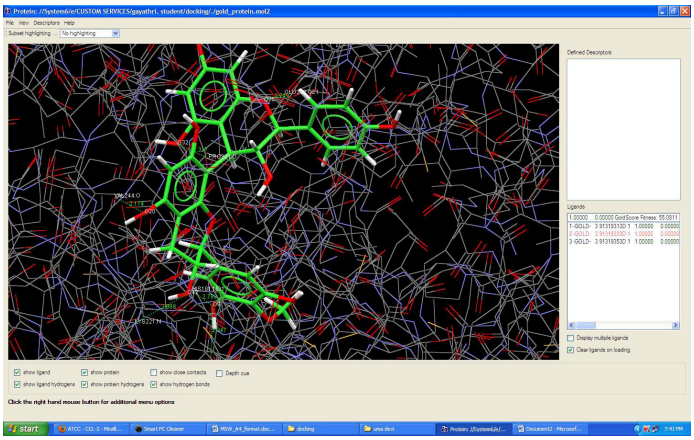

Figure 5: Molecular docking of procyanidin B_2 with NF-κB.

et al[22] reported the presence of monomeric flavonoids in *Musa sapientum* by using HPTLC techniques at 254 nm. In this study, the results of UV absorption and RP-HPLC monitored at 280 nm, showed the presence of phenolic compounds in methanolic extract of bark.

A total of 96 fractions were collected from the column chromatography and all the fractions were analysed for the presence of procyanidin B_2 by TLC and confirmed by dipping in vanillin-HCl Fractions in toluene:ethylacetate solvent gave positive result for the presence of procyanidin B_2 (Figure 3). Fractions which showed similar spots and Retardation factor (Rf) values were pooled. Thus, procyanidin B_2 rich fraction was got.

GC-MS analysis of procyanidin B_2 rich fraction

From the results given in Figure 4A and 4B, it is revealed that the procyanidin B_2 rich fraction contains dimeric proanthocyanidins.

From the results, it is revealed that the procyanidin B_2 rich fraction contains five major compounds with RT values of11.30, 11.66, 12.23, 12.52 and 12.65. The peak at RT 12.23 for procyanidin B_2 rich fraction was comparable with the peak at RT 12.25 obtained for standard procyanidin B_2.The results in this study, confirms the presence of dimeric proanthocyanidins in the bark extract.

Molecular docking of procyanidin B_2 against NF-κB.

The transcription factor NF-κB is an interesting target molecule for the design of lead compounds of anti-tumor, anti-inflammatory and pro-apoptotic drugs.[10] The best ligand docking gold score value was 55.03 with lowest binding energy of -6.03 (kcal/mol) and formed H-bonding with six amino acid residues (E 282, P283, V244, K221, H 181, I196) of p65 subunit of NF-κB (Figure 5 and Table 1).

In a mini review survey by Patrick,[18] showed the list of amino acid residues involved in interactions between IκBα and p50/ p65.The amino acids in p65subunit, Y20, E 22,E49, H181, K 221, S 238, F 239, S240, Q241, A 242, D 243, R 246, D 291, I295, K 298involved in binding with IκBα .

Thavamani *et al* [23] carried out molecular docking for 12 active compounds present in *Cocculus hirsutus* against hepato cellular carcinoma targets such as Aurora kinase, c-Kit, fibroblast growth factor, nuclear factor kappa B (NF-kB), B-cell lymphoma-extra-large, and vascular endothelial growth factor (VEGF) with the software Grid-Based Ligand. The results indicated that coclaurine, haiderine, and hisutine had good inhibition on NF-κB signaling pathway.

The present study shows the procyanidin B_2 formed H-bonding with H 181 and K 221 ofp65subunit of target protein. Hence, it supported the

Table 1:Atoms involved in H-bonding between ligandwith target protein for the best score

Ligand name	Protein name	Atom in ligand	Atom in protein	Hydrogen bond distance	Score
Procyanidin B_2	NfκB	O25	E282:OE1	2.749	55.08
		O32	P283:O	2.317	
		O20	V244:O	2.714	
		O42	K221:N	2.988	
		O9	H181:ND1	2.783	
		O9	I196:CB	2.347	

fact that the ligand is located in IκBα binding site of target protein. This shows that procyanidin B_2 could have the potency to mimic the action of IκBα.

Piccagli *et al.*[10] studied the amino acids of p50/p65 heterodimer involved in DNA binding and revealed that 13 p50 residues (C59, K144, Y57, Q274, Q306, R305, K145, K272,S63, G65, G66, N136, K77) and twelve p65 amino acids (C38, K122, Y36, K123, Q220, Q247,R246, K221, A 43, S42, S45, K56) made several contacts in DNA backbone.

Chen *et al.*[24] studied the crystal structure of p50/p65heterodimer of transcription factor NF-κB bound to DNA and showed that G65, G 66, G 88, H64, N54, N56, E 60, T67,K 241,C59, Y 57, K 144, K145, Q274, K 275, R 305 are the residues in p50 of p50/p65hetrodimer bind with DNA. Similarly R246, K 221, K218, E 220, E247, R246, Y36, C38,K 122, K 123 R 33, R 35, R187, and E 39 are the amino acid residues in p65 subunit of p50/p65hetreodimer bind with DNA.

In the present findings also, K221 formed H-bonding with procyanidin B_2 thus could interfere with binding of p50/p65 heterodimer to κB DNA binding sequence. This proved that the ligand is positioned in a cleft surrounded by active site amino acids of NF-κB. The hydrogen bonding plays the most important role in determining the specificity of intermolecular interactions. These findings supported that procyanidin B_2 by binding to active site of NF-κB could inhibit the nuclear translocation and DNA binding of p50/p65 heterodimer to κBDNA sequences. Thus, procyanidin B_2 can act as the inhibitor for transcription factor NF-κB.

CONCLUSION

Natural products play an important role in the novel drug discovery for the treatment of human diseases including cancer.[25] Chemical compounds derived from plants inhibit carcinogenesis through many mechanisms which include antioxidant activity, changes in carcinogen metabolism, modulation of cell cycle, alternations in their intracellular signaling and inhibition of angiogenesis.[26] So, procyanidin B_2 present in *C. zeylanicum* bark can be used as a potential lead compound for drug development against cancer and other oxidative stress disorders.

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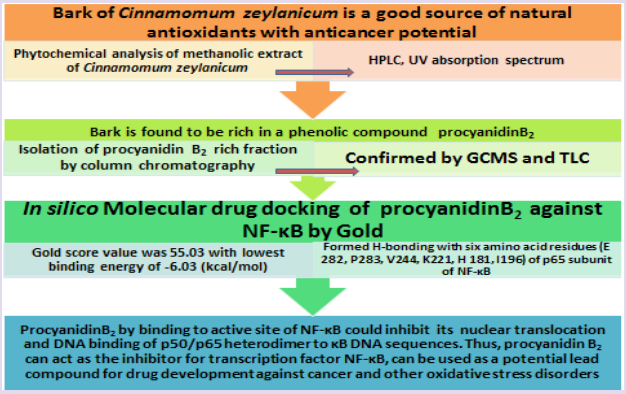
PICTORIAL ABSTRACT



SUMMARY

- The bark of *Cinnamomum zeylanicum* is rich in medicinally essential phytoconstituents especially phenolic compounds.
- The methanolic extract is found to be rich in procyanidin B_2
- *In silico* Molecular drug docking of procyanidin B_2 against NF-κB by Gold
- Gold score value was 55.03 with lowest binding energy of -6.03 (kcal/mol)
- Formed H-bonding with six amino acid residues (E 282, P283, V244, K221, H 181, I196) of p65 subunit of NF-κB
- Thus, procyanidin B_2 can act as the inhibitor for transcription factor NF-κB, can be used as a potential lead compound for drug development against cancer and other oxidative stress disorders.

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