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Trigonella seed extract ameliorates inflammation via regulation of the inflammasome adaptor protein, ASC

Nidhi Sharma[1], Samyuktha Suresh[2], Ananya Debnath[3], Sushmita Jha[1]

[1]Department of Biology, Indian Institute of Technology Jodhpur, Old Residency Road, Ratanada, Jodhpur, Rajasthan, India 342011, [2]Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur, Chennai, Tamilnadu, India 603203, [3]Department of Chemistry, Indian Institute of Technology Jodhpur, Old Residency Road, Ratanada, Jodhpur, Rajasthan, India 342011

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1. ABSTRACT

Trigonella foenum-graecum (fenugreek) is an important medicinal plant, well known for its anti-inflammatory properties. However, the underlying cellular and molecular mechanisms of its action remain largely unknown. The apoptosis associated speck like protein containing a caspase recruitment domain (CARD) (ASC) is central to inflammatory and cell death pathways in innate and adaptive immunity. Here, we show that fenugreek seed extract provides cytoprotection to bacterial lipopolysaccharide (LPS) inflammed and nanosilica-treated fibroblasts via a reactive oxygen species independent pathway. All atom molecular dynamics simulations of ASC-ligand complex reveal that individual phytochemicals in fenugreek can bind to ASC via specific non-covalent interactions. These data show that a synergistic effect of fenugreek phytochemicals with the ASC protein alters its molecular properties resulting in altered cellular function. Such information is crucial to the development of targeted therapeutic interventions for inflammatory diseases.

2. INTRODUCTION

Inflammation is a complex biological response to injury, pathogens, or irritants. Dysregulated inflammation is central to seemingly unrelated diseases such as; cancer, autoimmune diseases, allergy, metabolic and cardiovascular disease. *Trigonella foenum-graecum* or fenugreek is widely accepted as an anti-inflammatory agent with cytoprotective effects. However, the underlying cellular and molecular mechanisms of fenugreek-mediated cytoprotection are largely unknown (1). Fenugreek extracts act as potent anti-microbial and anti-inflammatory agents (2, 3). These extracts are beneficial against diabetes, cancer and hypercholesterolemia (4-6). Some of the important phytochemicals in fenugreek include trigonelline, diosgenin, tigogenin, luteolin-7-O-glucoside (henceforth, referred to as luteolin), kaempferol and apigenin. In a rat model of diabetes, the hydro-alcoholic extract of fenugreek, reduced inflammatory markers including; tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (7). A similar reduction in the levels of TNF-α, IL-1α, IL-1β, IL-6 was observed when rats injected with Freund's complete adjuvant were treated with fenugreek extract (8). Additionally, the levels of IL-4, an anti-inflammatory cytokine, were restored with fenugreek treatment (3). While there are many studies describing the cytoprotective and anti-inflammatory effects of various fenugreek phytochemicals and extracts, there is a dearth of mechanistic insight.

The NLR (nucleotide-binding oligomerization domain receptors) family of proteins acts as pattern recognition receptors in innate and adaptive immunity (9). Dysregulated NLR signaling is central to the pathology of several inflammatory and autoimmune diseases including cardiovascular disease, diabetes, multiple sclerosis and cancer (10). NLRs, aid in recognition of damage associated molecular patterns (DAMPs) such as; ATP, uric acid, amyloid -β, hyaluronan and heparan sulfate, pathogen associated molecular patterns (PAMPs) such as; bacterial and viral nucleic acids and irritants such as; silica, asbestos and alum.

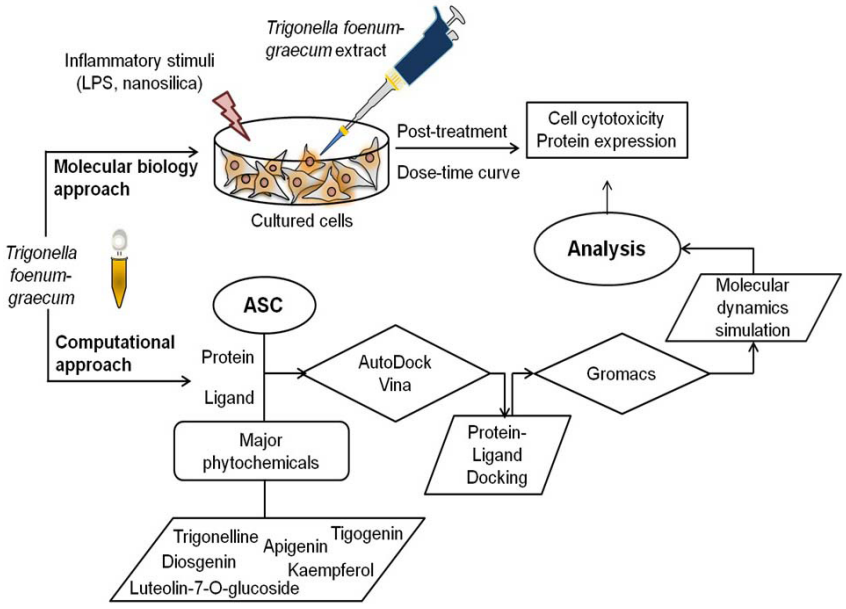

Figure 1. Analytic workflow for study of fenugreek-induced anti-inflammatory effects and possible interactions with ASC protein targeting *in-vitro* and *in- silico* approaches

On sensing DAMPs, PAMPs or irritants, NLRs associate with the adapter protein, Apoptosis associated speck like protein containing CARD (ASC) and procaspase-1 to form multi-protein complexes called *inflammasomes.* ASC is central to several inflammatory and cell death-associated pathways in innate and adaptive immune responses (11). ASC consists of a PYRIN domain at its N-terminus and a CARD at its C-terminus. The PYRIN domain belongs to the DD (Death domain) superfamily and is essential for protein interactions associated with apoptosis, inflammation and innate immune signaling pathways. The ASC PYRIN domain comprises of six anti-parallel helices. The PYRIN domain interacts through homotypic protein-protein interactions, contributing significantly to the regulation of inflammasome signaling. ASC utilizes its CARD domain to recruit pro-caspase-1 via homotypic interactions. In the inflammasome complex, the inactive procaspase-1 undergoes autocatalytic cleavage to form active caspase-1 (12). The activated caspase-1 in turn can cleave and activate more than 70 substrates, ranging from chaperones, cytoskeletal and translation machinery, glycolysis proteins to immune proteins such as the proinflammatory cytokines; IL-1β and IL-18 (13, 14). Stimuli that induce the activation of caspase-1 to process cytokines do not necessarily cause cell death (15).

Computational methods such as molecular docking and molecular dynamics simulation have been employed in association with atomic force microscopy (AFM) and solution nuclear magnetic resonance (NMR) to investigate the binding modes and interactions of fenugreek phytochemicals with the enzyme aldol reductase for its role in hyperglycemia (16). Both PYRIN and CARD domain of ASC are independent domains, connected by a linker with a residual structure favoring a back-to-back structure of domains. This topology avoids steric interference of each domain with the binding sites of the other and facilitates a specific protein binding partner (17, 18). The charged and hydrophobic residues contribute significantly to the nature of helical interactions between PYRIN domains (19-21). The helices can open up and expose the hydrophobic residues resulting in a symmetric dimerization mode revealed by the X-ray structure (22). Intrinsic flexibility of the PYRIN domain is found to be a key factor in influencing their function under physiological conditions (23). In fact, particular protonation states of acidic side chains are crucial for stabilizing the tertiary structure of ASC by forming salt bridges at low pH (24). Molecular simulations reveal that unlike NLRP1, the ASC PYRIN domain has a six-helix bundle structure with a specific loop between helix 2 and 3 without a significant increment in internal motion (25, 26). In support of the structure-function relationship of ASC PYRIN domain with its ligands, the current study sheds light on the molecular interactions of fenugreek with ASC to mediate cytoprotection in fibroblasts. The present study sought to assess fenugreek-mediated regulation of ASC expression during inflammation in fibroblasts.

The effect of fenugreek extract on nanosilica mediated cytotoxicity and bacterial lipopolysaccharide (LPS) mediated inflammation (27, 28) was investigated using experimental biology and molecular dynamics (Figure 1). Such an interdisciplinary approach allowed us to understand the cellular as well as molecular

effects of fenugreek on inflammation. We provide evidence that fenugreek regulates nanosilica mediated cytotoxicity via the interaction of the constituent phytochemicals with the inflammasome adaptor protein, ASC. While the cellular studies provide insight into the amelioration of cell death by fenugreek, the molecular dynamics analyses reveal specific residues and nature of interactions underlying the same allowing for the use of fenugreek in development of targeted therapeutics for inflammation.

3. MATERIALS AND METHODS

3.1. Cell culture

Chinese hamster ovary cells (CHO) were cultured in DMEM, high glucose medium, supplemented with 10% Fetal Bovine Serum and 1 % antibiotic antimycotic stabilized solution. Cells, culture media and supplements including DMEM, high glucose (AL007S), fetal bovine serum (RM10432) and antibiotics antimycotic solution (A5955) were purchased from Himedia (India).

3.2. Trigonella foenum-graecum (fenugreek) seed extract

The fenugreek seed extract (Viscous liquid; aqueous soluble) was purchased from Sigma-India. (Grade - Kosher, Natural, W248606). The anti-cytotoxicity and anti-inflammatory effects of fenugreek upon nanosilica-induced toxicity were measured using various concentrations (0-3000µg/mL) of fenugreek for 0-12 hour exposure times (Supplementary Figure- 1B).

3.3. Nanosilica exposure

CHO cells were primed overnight with 0.5. µg/mL of LPS from *Escherichia coli* 026: B6 (Sigma, India, L4391) (29, 30). After LPS stimulation, cells were washed with serum free media. Fresh serum free media was added before exposure to nanosilica. Ludox® silica nanoparticles, HS-40 (420816; Sigma) with an average size of 12nm were used. The nanosilica exposure was optimized at 200µg/mL for 60 minutes, after exposure time and dose-dependent analysis (Supplementary Figure- 1A).

3.4. ROS inhibition

N-acetyl-L-Cysteine (NAC) (Sigma, India, A9165) treatment for 20 minutes was used to inhibit ROS production as described previously (31).

3.5. Cytotoxicity screening

The cell cytotoxicity assessment was performed using MTT assay (Sigma, India, M5655) as per manufacturer's instructions.

3.6. ASC Immunocytochemistry

Cells were stained for ASC (Cell Signaling Technologies, India, Rabbit mAb, 13833S) as described previously (32). 4', 6'-diamidino-2-phenylindole (DAPI) (Sigma, India, F6057) was used to stain nuclei blue. Immunopositive cells with an observable DAPI stained nucleus were counted blindly twice. ASC speck quantification was done using ImageJ (NIH, free license) software.

3.7. Simulation method

The phytochemical groups chosen for molecular simulation studies of fenugreek-ASC complex are listed in Table 2. To obtain the initial configurations of fenugreek-ASC complex for molecular dynamics simulations, molecular docking tool - AutoDock Vina was employed (33). ASC PYRIN domain structure was obtained from NMR (PDB ID: 1UCP). Since polar hydrogen has electrostatic importance during docking, it was added before the simulation if not present in the PDB file. The structure files of ligands (Table 2) were downloaded from PubChem (tigogenin - CID99516, apigenin- CID5280443, trigonelline - CID5570, diosgenin- CID99474, kaempferol- CID5280863, luteolin- CID5291488). The fenugreek-ASC complex (individual phytochemicals of fenugreek with ASC) was docked to generate different conformations. Since the docked conformations show the most suitable orientation of ligands to ASC with strong binding modes (without the effect of the environment), these were used as the starting configuration of molecular dynamics simulations (34). The simulations were carried out using the GROMOS53a6 force-field parameters (35) for the protein and the SPC model for water (36). The force-field parameters for the ligands were generated from Automated Topology Builder (ATB) and repository (37-39). Seven sets of simulations were carried out where one set includes only ASC protein and the other six sets include ASC protein with six individual fenugreek phytochemicals as ligands. All systems were solvated with shell of water molecules and two molecules of sodium ions were added to neutralize each system. The systems were energy minimized using the steepest descent algorithm, followed by a 100ps NVT run using V-rescale temperature coupling method (40) with a coupling constant of 0.1.ps. 100ps NPT runs were carried out using Berendsen thermostat (41) with a coupling constant of 0.1.ps by Parrinello-Rahman method (42).

4. RESULTS

4.1. Fenugreek extract ameliorates lipopolysaccharide and nanosilica mediated cell cytotoxicity

ASC is essential for LPS-induced activation of procaspase-1 independently of TLR-associated

Table 1. ASC protein residues forming hydrogen bonds or contacts with the individual phytochemical ligands

Phytochemical ligands	Residues of ASC protein	
	Hydrogen bonds	Contacts
Apigenin	38ARG, 41ARG, 32LEU, 33ARG, 29SER, 34GLU, 30VAL, 35GLY, 37GLY, 36TYR, 39ILE	*34GLU, 32LEU, 38ARG, 29SER, 41ARG, 35GLY, 37GLY, 36TYR, 33ARG, 39ILE 30VAL*
Trigonelline	38ARG, 41ARG, 29SER, 26LYS, 1MET, 37GLY, 42GLY, 43ALA, 64TYR, 71ASN	*37GLY, 38ARG, 29SER, 1MET, 26LYS, 71ASN , 41ARG,* **46SER**, *42GLY, 64TYR, 43ALA*
Diosgenin	3ARG, 84GLN, 7ALA, 6ASP,10ASP	**19GLU**, *84GLN*, **16THR**, *10ASP, 7ALA, 3ARG, 6ASP*,
Luteolin	33ARG, 31PRO, 64TYR, 36TYR, 89THR, 71ASN, 91GLN, 67GLU	*31 PRO, 33ARG, 64TYR, 89THR, 71ASN, 67GLU 36TYR, 91GLN*
Kaempferol	33ARG, 67GLU, 71ASN, 75ASP, 26LYS, 29SER, 64TYR, 31PRO	*67GLU, 75ASP, 33ARG , 29SER, 71ASN, 64TYR, 31PRO, 26LYS*
Tigogenin	6ASP, 10ASP, 7ALA, 84GLN, 3ARG	**16THR**, **19GLU**,**14ASN**, *84GLN, 10ASP, 7ALA, 3ARG, 6ASP*

The contact residues which are also forming hydrogen bonds are shown in italics while other residues forming contacts are in bold

Table 2. Major phytochemicals present in the fenugreek seed extract

Major nutrients	Phytochemicals
Alkaloids	Trigonelline
Saponins	Tigogenin
	Diosgenin
Flavonoids	Apigenin
	Kaempferol
	Luteolin

signal adaptor molecules (43). Chinese hamster ovary (CHO) fibroblast cells were stimulated overnight with bacterial lipopolysaccharide (LPS: 0.5. µg/mL) to induce inflammation. Following LPS stimulation, fibroblasts were then treated either with nanosilica (200 µg/mL), to induce cell death, or a combination of nanosilica and fenugreek, to evaluate the effect of fenugreek on nanosilica mediated inflammation and cell death. After the dose-response analysis, fenugreek concentrations of 800-3000 µg/mL were utilized for 60 minutes. Morphological analysis using phase contrast microscopy showed that nanosilica induced cell death is significantly reduced when the cells are treated with fenugreek extract (Figure 2 (B, E)).

The cytotoxicity data analysis shows that fibroblasts treated with nanosilica and LPS undergo rapid cell death but are protected in the presence of fenugreek seed extract in a dose dependent manner in the presence (Figure 3B) and absence of LPS induced inflammation (Figure 3A). We next investigated if fenugreek treatment prior to nanosilica exposure would provide protection from nanosilica induced cytotoxicity. However, pretreatment with fenugreek extract, 6 hours and 12 hours, prior to nanosilica exposure did not prevent nanosilica induced cytotoxicity (Figure 3 (C, D)). Suggesting that the presence of phytochemicals in the fenugreek extract and not cellular mechanisms elicited by fenugreek exposure are involved in fenugreek-mediated protection from nanosilica-induced cell death.

4.2. Mechanism of Fenugreek mediated suppression of cytotoxicity is independent of reactive oxygen species

Free radicals are implied in a number of diseases and health conditions like cancer, chronic inflammation and neurodegenerative diseases. Fenugreek seed extract is also known to scavenge free radicals (44). Fenugreek seeds restore the levels of anti-oxidants such as; glutathione (GSH) and Ascorbate, anti-oxidant enzymes such as; glutathione peroxidase (GPx), glutathione-S transferase (GST), catalase (CAT) and superoxide dismutase (SOD) and oxidative stress markers such as malondialdehyde (2, 3, 45). Mucilage extracted from fenugreek reduces the levels of inflammatory markers such as cyclooxygenase-2 (COX-2), lipoxygenase-2, myeloperoxidase, C-reactive protein and nitrite in adjuvant induced arthritis in rats (2). Next, to investigate if fenugreek seed extract provides protection against nanosilica by suppression of reactive oxygen species (ROS), we utilized *N*-Acetyl-l-cysteine (NAC), a ROS scavenger (46). Interestingly, NAC could not rescue nanosilica-induced cell death suggesting that here fenugreek mediated cytoprotective

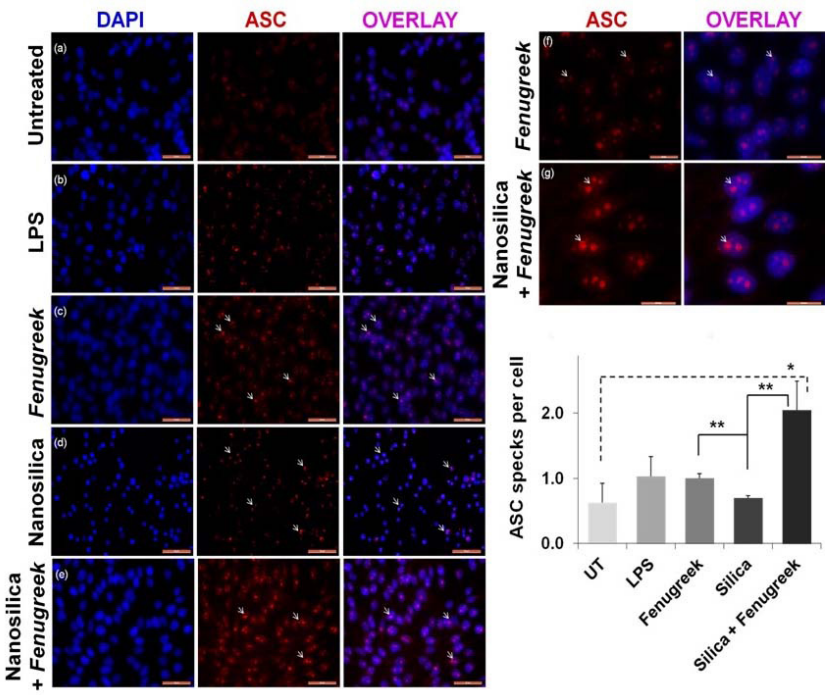

Figure 2. Inhibitory effect of fenugreek on nanosilica-induced cytotoxicity. The above panel shows reduced cytotoxicity and cell death in LPS-primed fibroblast cells upon exposure to nanosilica and fenugreek *(*B-E). Scale bar: 100µm. (F) ASC expression visualized in fibroblast cells exposed to fenugreek along with nanosilica. Right panel - ASC expression in control (a), LPS treated (b*)*, fenugreek treated (c), nanosilica treated (d) and nanosilica and fenugreek treated (e) fibroblast cells are visualized by using immunofluorescence microscopy for ASC (red) and DAPI to stain nuclei blue. Scale bar-50µm. The right panel shows inset images of fenugreek treated (f) and both nanosilica and fenugreek treated (g) fibroblast cells respectively at higher magnification. Scale bar-20µm. Graph shows ASC speck quantification in LPS-primed, silica and fenugreek treated cells. Data is presented as mean ± SD; *p value < 0.0.5., **p value < 0.0.0.5. by t-test analysis

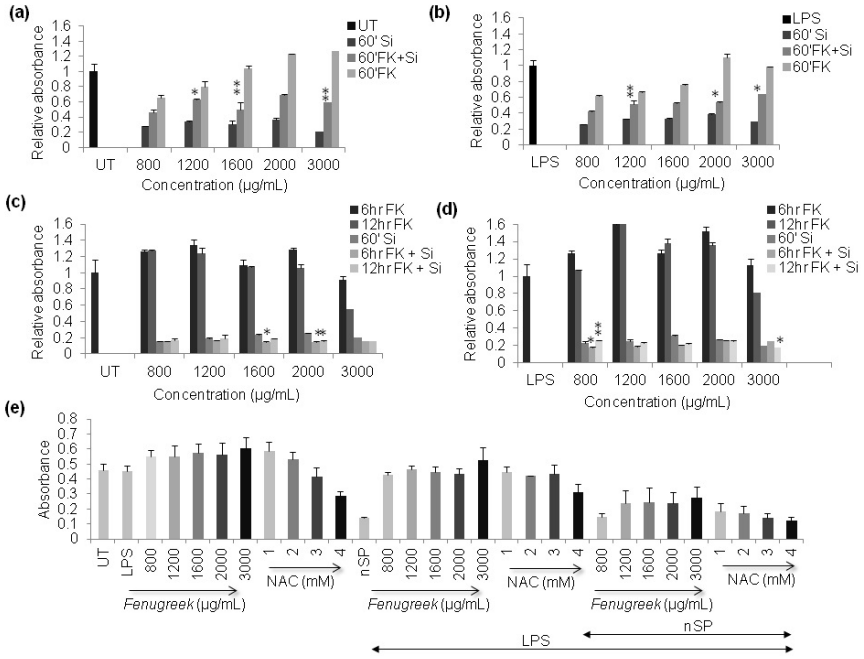

Figure 3. Effect of fenugreek seed extract on nanosilica-induced cytotoxicity. Dose-dependent decrease in cytotoxicity caused by nanosilica (200 µg/mL) was observed in presence of fenugreek both in untreated fibroblasts (A) and LPS-primed (0.5. µg/mL) fibroblasts (B). Pretreatment of fibroblasts with fenugreek for 6h or 12h prior to nanosilica exposure did not offer any cytoprotection both in the absence (C) and presence of LPS priming (D). Pretreatment with NAC (1-4mM) did not inhibit nanosilica-induced cytotoxicity.Instead, resulted in increased cytotoxicity levels in dose-dependent manner (E). Data is presented as mean ± SD; *p value < 0.0.5., **p value < 0.0.0.5. by t-test analysis

mechanism must be independent of reactive oxygen species (Figure 3E).

4.3. Expression of ASC protein increases following fenugreek treatment

LPS induces formation of ASC supramolecular assembly referred to as apoptotic speck or pyroptosome (47). In the bone marrow derived macrophages, ASC speck formation is required for processing of IL-1β but dispensable for pyroptosis induction. ASC oligomerization creates multiple caspase-1 activation sites, serving as a mechanism for signal amplification of inflammasome dependent cytokine production (48). To assess ASC protein expression following fenugreek treatment, we carried out immunofluorescence for ASC (Figure 2F (a-g). The cytoplasmic and diffuse expression of ASC in untreated fibroblasts increases upon LPS stimulation. Nanosilica treated cells undergo rapid cell death, show nuclear condensation and presence of cytoplasmic as well as perinuclear specks. Strikingly, fenugreek and nanosilica treated fibroblasts remain viable, show absence of nuclear condensation and increased ASC expression with several cytoplasmic and perinuclear specks (Figure 2- Graph).

4.4. Interaction of ASC protein with individual fenugreek phytochemicals

In silico approaches were utilized to gain molecular insights into the cytoprotective effect of fenugreek. All atom molecular dynamics (MD) simulations of individual fenugreek phytochemicals (trigonelline, diosgenin, luteolin, kaempferol, tigogenin and apigenin) with ASC were carried out to observe protein-ligand interactions. ASC interacts with NLRP3 via homotypic PYRIN-PYRIN interactions and recruits procaspase-1 via homotypic CARD-CARD interactions. Therefore, both PYRIN and CARD domains were docked with individual phytochemicals. Since the PYRIN domain expressed higher affinity towards ASC ligand binding as observed in previous reports (25), we used this domain for MD simulations with preferred orientations of the individual fenugreek phytochemicals as obtained from docking studies. Fluctuations of the protein residues due to ligand binding were identified by root mean square fluctuations (RMSF) of protein residues. The RMSF values were calculated after a least square fitting to a reference frame of starting structure of the 30 ns trajectory. The RMSF for ASC protein residues both in the absence and presence of six ligands are shown in Figure 4a. Higher fluctuations were observed for residues in the loop regions and in the 1st and 5th helices, in case of apigenin, kaempferol, trigonelline and luteolin. This indicates that ASC does not change its global structure while interacting with the individual phytochemicals and the probable locations of the binding domains are either in the loop regions or in the 1st or 5th helices.

The root mean square deviations (RMSD) of the protein was calculated after least square complete fitting (translation and rotation) of the Cα atoms to their corresponding initial structures of the production runs (Figure 4b). In the presence of kaempferol, apigenin and trigonelline, the RMSD values of ASC are enhanced as compared to the initial configuration, but not significantly high enough to predict any conformational change in protein due to ligand binding. This reaffirms that ASC does not deviate from its reference conformation to bind these phytochemicals.

To identify the interactions between fenugreek ligands and ASC protein, probability of hydrogen bond formation is calculated between all possible donors and acceptors of the protein and the individual ligands, using well-established geometric criteria: $r \leq 3.5$A°, $\alpha \leq 30°$, where r is the distance between donor and acceptor and α is the angle between hydrogen donor and acceptor. Figure 5a describes the distribution of hydrogen bonds with respect to the number of hydrogen bonds between individual phytochemical ligands and the ASC protein. The plot clearly shows that all ligands participate in hydrogen bonding where luteolin-bound ASC forms the highest number of hydrogen bonds. Minimum distance calculation was done between individual ligands and ASC with a distance cut-off of 0.3. nm (data not shown). Table 1 shows the list of residues that form contacts with the protein due to non-covalent interactions which can be ion-dipole, dipole-dipole or hydrogen bonding. Most of the protein contacts are formed by the same residues that participate in hydrogen bonding with the ligands (shown in italics, Table 1). Only trigonelline, diosgenin and tigogenin formed few additional contacts with the protein which are not part of the hydrogen bonds. These contacts are formed either by a charged residue (GLU) or a polar residue (THR/ASN) of the protein due to either ion-dipole or dipole-dipole interactions (Table 1). Several of the interacting residues suggested by our studies are identical or adjacent to residues essential for filament formation and oligomerization of ASC as reported earlier (19).

The residues that form H-bonds or contacts (Table 1) with six phytochemical ligands are located either in the loop region between the 2nd and the 3rd helix or at the initial or end points of the helices of ASC (Figure 5b). Also, no ligands are sandwiched between two helices to interact with the protein residues (Figure 5(c-h)). This interaction scenario is possible, when two helices are located back-to-back in such a way that the inner surface between two helices are not perturbed by the presence of the ligands and the outer surface is open for the ligand binding through the regions near the loops or bends. The interacting residues of the helices mostly occur in the 1st and 5th helices (C-terminal side), which is consistent with the higher RMSF values of the N/C terminal residues (shown in Figure 4a). The higher

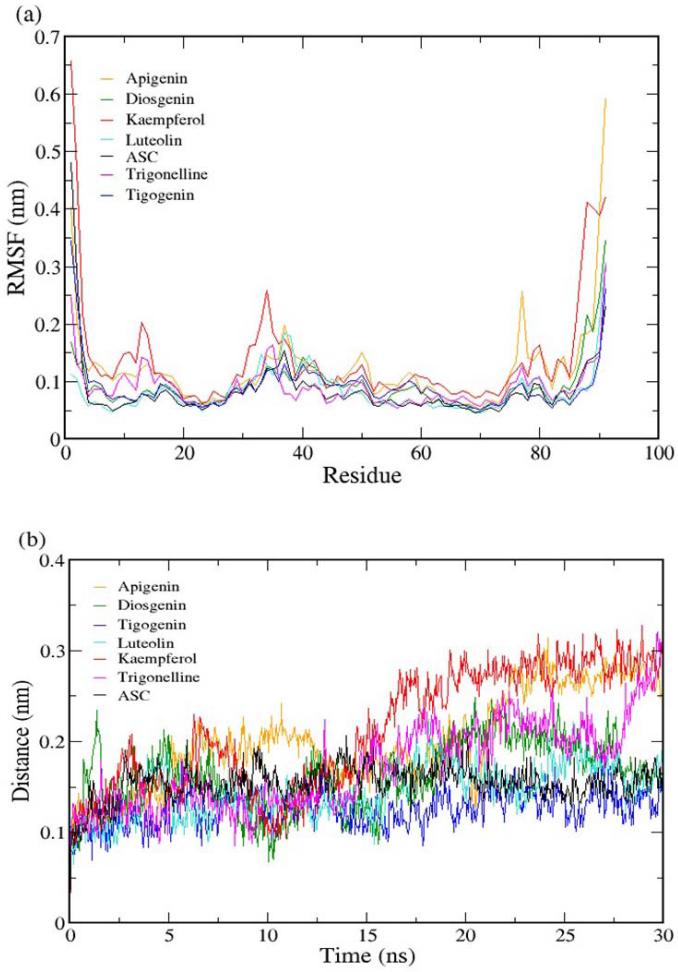

Figure 4. (a) Root mean square fluctuations of ASC protein residues. (b) Root mean square deviation of ASC protein are shown with respect to simulation time. The graphs show ASC protein residues without any ligand (black) and in the presence of six individual ligands (Apigenin: orange, Diosgenin: green, Kaempferol: red, Luteolin: turquoise blue, Trigonelline: magenta, Tigogenin: blue)

fluctuations of the residues in between N/C termini occur in the loop or bend regions to form h-bonds or contacts with the ligands. The RMSD of the protein in the presence of apigenin, kaempferol and trigonelline are above 0.2.5 nm since the residues (Table 1) forming h-bonds/contacts with these three ligands reside mostly in a loop or partly in all helices except the 4th one. However, a secondary structure analysis of the protein in the presence of these ligands reveals that the helical domains (2nd, 3rd and 5th helix) do not unfold during the interactions (data not shown). Therefore, to bind with these ligands, the protein has to deviate from its reference structure without any unfolding of these helices.

A comparative analysis of the fluctuations of ASC (RMSD and RMSF) protein residues in the presence and absence of phytochemical ligands, suggests possible interactions between the ASC and phytochemicals. In the presence of phytochemicals, ASC depicts higher RMSD only in the loop and the N/C terminal domains due to their flexible and exposed nature, whereas rests of the helical residues remain stable with a RMSD value of 0.1.5 nm. The RMSF of ASC reaches a plateau below 0.2. nm for all ligands except kaempferol, apigenin and trigonelline where the fluctuations are little enhanced (near 0.3. nm). However, no significant fluctuations are observed which may indicate an unraveling of folded conformation of ASC to bind these specific ligands. Our simulations demonstrate that ASC binds to all individual phytochemical ligands via specific non-covalent interactions such as hydrogen bonding, where luteolin forms the highest number of hydrogen bonds. Moreover, few contacts have been found between ASC - diosgenin and - tigogenin in addition to the hydrogen bonds where ion-dipole or dipole-dipole interactions might be the stabilizing factors. A comparison of the secondary structure sequence and the location of hydrogen bond or contact forming residues (Figure 5 (b-h)) reveal that the binding sites mostly occur in the loop regions or at the start or end points of all

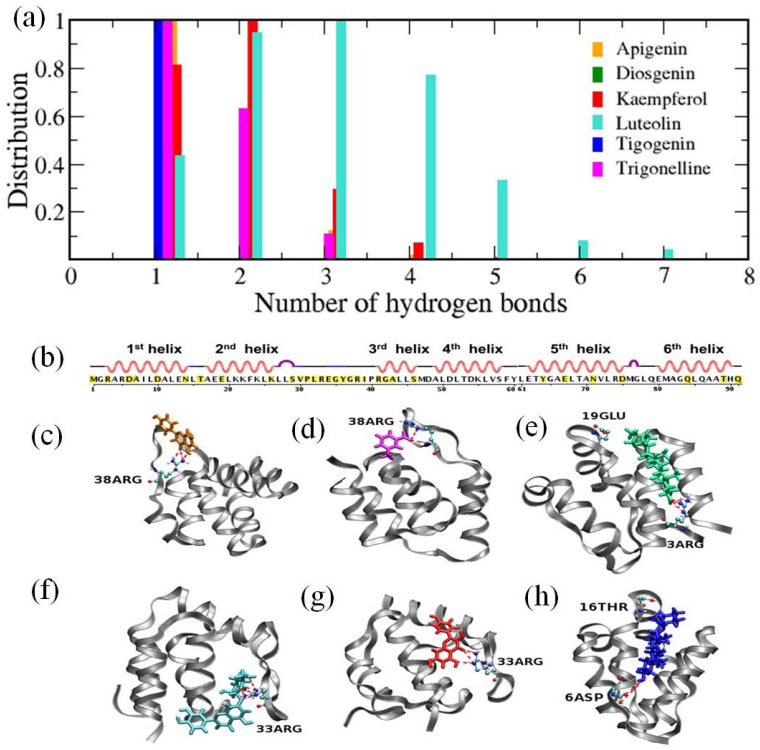

Figure 5. (a) Hydrogen bond distribution between individual phytochemical ligands and ASC protein. (b) Sequence of ASC PYRIN domain shows 6 helical regions. Yellow blocks denote the residues interacting with six phytochemical ligands. (c-h) Snapshot depicting interactions between ASC protein and phytochemical ligands

helices except the 4th helix. Although these interacting domains have enhanced RMSD values, they do not change their secondary structures significantly.

5. DISCUSSION

Although specific non-covalent interactions are found to be responsible for individual phytochemical-ASC binding, the structure of ASC remains unaltered. Interestingly, none of the ligand's interaction with ASC can be held solely responsible for the cytoprotective effects of fenugreek. This is also supported by the earlier findings where fenugreek crude seed extract was selectively toxic to cancer cell lines and not harmful to the normal counterparts but purified components such as diosgenin were toxic to both normal and cancer cell lines (49). This selective cytotoxicity was also observed in the oil extracted from fenugreek seeds (50). Recently, Dick *et al.*, reported the mechanism of ASC filament formation and signal amplification for inflammasome mediated cytokine production (48). The study provides evidence that formation of ASC specks requires the entire PYRIN domain. Importantly, their mutational analyses confirm, the residues listed by us as critical for interaction with fenugreek phytochemicals are important for inflammasome signaling but not cell death. For example, the K26 residue interacts with trigonelline and kaempferol and is essential for speck formation. The Y36 residue that interacts with apigenin and luteolin has an intermediate role in speck formation while the R41 residue that interacts with Apigenin and Trigonelline is important for induction of downstream inflammasome signaling.

Endogenous decoy proteins containing only the PYRIN domain (POPs) are suggested to interfere with NLRs and ASC interaction thus preventing inflammasome activation. Srimathi *et al*, mapped the binding site of PYRIN domain-only protein 1 (POP1) on ASC (51). Interestingly, Y36 and R41 are listed as important for interaction of POP1 with ASC (52). *En toto* these findings suggest a cumulative interaction of fenugreek phytochemicals with the ASC protein to affect change in the molecular properties resulting in altered cellular function. The interaction of ASC PYRIN domain with the phytochemicals raises possibility of fenugreek components affecting ASC activity by modulating its structure or function. These interactions might also introduce regulatory changes in ASC-associated inflammation and cell death pathways. In this respect, our present study lays the foundation for a future investigation of synergetic effect of fenugreek-mediated regulation of ASC, which may

offer an opportunity for development of therapeutic interventions for many inflammatory diseases by establishing their structure-function relationship.

6. ACKNOWLEDGEMENTS

SJ conceived the study; AD and SJ supervised the study; SS and NS performed molecular dynamics simulations; NS performed cell biology experiments; AD, NS and SJ wrote the manuscript. The authors have no conflict of interest. SJ's laboratory is funded by grants from the Department of Science and Technology (Young scientist scheme, SB/YS/LS-282/2013) and Board of Research in Nuclear Sciences (2013/36/72-BRNS/2415), Government of India. The software application Science Slides (VisiScience) was used to generate parts of Figure 1. Ananya Debnath is thankful to IIT Jodhpur high performance cluster facility for computational work.

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Abbreviations: ASC: Apoptosis-associated speck-like protein containing Caspase recruitment domain, CARD: Caspase recruitment domain, LPS: bacterial lipopolysaccharide, RMSF: Root mean square fluctuations, RMSD: Root mean square deviations

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Send correspondence to: Sushmita Jha, Department of Biology, Indian Institute of Technology Jodhpur, Old Residency Road, Ratanada, Jodhpur, Rajasthan, India 342011, Tel: 91-291- 244-9083, Fax: 91-291-251-6823, e-Mail: sushmitajha@iitj.ac.in