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Bioactive Compound of *Citrus reticulata* Support the TNF – α in Tissue Proliferation

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ABSTRACT:

TNF-α plays an important role in the healing of wound by angiogenesis and granulation of tissue at the site of the wound. In this study we have studied this protein TNF- α from its structure perspectives. Its primary and secondary structures were evaluated using online tools. Its role in wound healing was assessed by docking the compounds present in the peel extract of *Citrus reticulate* assayed by GC-MS analysis compared to Nitrofurazone (the standard drug). The results throw light on how these compounds are effective in wound healing.

Keywords: TNF α, Tissue proliferation, Docking studies, GC-MS, *Citrus reticulata*

INTRODUCTION

Wound healing involves various phases such as Homeostasis, Inflammation, Proliferation, Platelet Aggregation and Remodeling. These various phases are regulated by different factors such as cytokines and growth factors [1]. Growth factors such as FGF, PDGF, EGF, VEGF, TGF β and cytokine of TNF α, are reported to be involved in various biochemical actions [2]. Among these, TNF- α is associated with persistent inflammation and tissue destruction [3-5]. It is produced from different types of cells such as macrophages, T cell, Mast cells and Keratinocytes [6].

It has two types of receptors i.e. TNF-R_p55 have molecular mass 55kDa and TNF-R_p75 have 75kDa molecular mass [7]. These receptors have 30% homology at the amino acid level in extra-cellular region and ligand binding region and have more amount of cysteine.

TNF-R_p55 is expressed on all types of cells except RBC [6], whereas TNF-R_p75 expression was restricted in hematopoietic and endothelial cells. TNF-R_p55 involves different biological activities of TNF-α such as cytotoxicity and fibroblast proliferation [8].

In our study the structure of TNF- α was computed by various bioinformatics tools and various physiochemical parameters such as Aliphatic index, Gravy, Isoelectric point (pI), Extinction coefficient (EC) were evaluated systematically. Their functional features and amino acid sequence were used to determine the characterization of the molecule, and pockets which was ready to binding.

It has been a tradition to use plants for treatment of various diseases. Local application of the some plant extracts is used by various tribes in India. However, these do not have any scientific basis as to how the wound healing takes place. In this study we have evaluated the role of compounds present in the peel extract of *Citrus reticulate* in wound healing.

[II] MATERIALS AND METHODS

2.1. Protein sequences

Tumour necrosis factor of preotein sequence was retrived from the online database of SWISSPROT [9]. It accesses the input keyword of TNF alpha and search in the entire database. A number of TNF-α sequence were shown. Among which Human TNF-α was accessed in FASTA format and it was used for the further computational analysis.

2.2. Primary structure analysis: Expasy protparam(http://us.expasy.org/tools/protparam.html) prediction server used to determine the primary structure and their physiochemical parameters such as amino acid composition, theoretical isoelectric point(PI), molecular weight, total number of positive and negative residues, extinction coefficient [10], Half- life [11, 12, 13, 14, Instability index [15] and Grand Average Hydropathy (GRAVY) [16].

2.3. Secondary Structure Prediction: SOPMA, SOPM [17] and SSCP server [18] were used to predict the secondary structure of TNF α.

2.4. Transmembrane Region Identification: The identification of transmembrane region of TNF-α was performed by using SOSUI [19]. The evaluated transmembrane region analysed and visualized by Pepwheel [20] using EMBOSS 2.7 suite.

2.5. Homology modeling and validation: SWISS-MODEL [9] used to determine the 3D structure of TNF-α and evaluated by Rampage [21] online server. The protein was validated by using online server Prochek [22] and WHAT IF [23].

2.6. Pocket Finder: TNF- α sequence induced to illustrate the number of pockets which was ready to binding ability, was analysed by using the server pocket finder [24].

2.7. GC-MS Analysis: *Citrus reticulata* peel extract undergoes with GC-MS analysis and identified the distinguished compounds.

2.8. Ligand Retrived: The identified compounds and the standard drug were retrived from the pubchem compound (http://www.ncbi.nlm.nih.gov/pccompound) and used for the further studies.

2.9. Receptor retrived: The receptor of TNF-α protein was downloaded from the PDB (http://www.rcsb.org/pdb/home/home.do) and the PDB ID: 1TNF.

2.10. Docking studies: Docking calculations were carried out using Docking Server [25]. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools. Affinity (grid) maps of ×× Å grid points and 0.375 Å spacing were generated using the Autogrid program [26]. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method [27]. Initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released

during docking. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

[III] RESULTS

3.1. Primary structure analysis:

Human TNF-α (P01375) had gene name as TNF TNFA TNFSF2 and the term Tumor necrosis factor used as protein, was retrived from SWISS PROT as the form of FASTA sequence.

FASTA Sequence:

MSTESMIRDVELAEEALPKKTGGPQGSRRC
LFLSLFSFLIVAGATTLFCLLHFGVIGPQREE
FPRDLSLISPLAQAVRSSSRTPSDKPVAHVV
ANPQAEGQLQWLNRRANALLANGVELRD
NQLVVPSEGLYLIYSQVLFKGQGCPSTHVL
LTHTISRIAVSYQTKVNLLSAIKSPCQRETPE
GAEAKPWYEPIYLGGVFQLEKGDRLSAEIN
RPDYLDFAESGQVYFGIIAL

The primary physiochemical parameters performed and aminoacid composition were identified; the details were shown in Table 1 and 2. The result represented that the TNF-α proetein composed of 22 aminoacids with different ratio. Among that leucine content was more (12.9%) that inidcate the hydrophobic nature of protein because it has an aliphatic isobutyl side chain and also essential aminoacid. The molecular weight of protein was found to be 25644.4, the protein has 6.44 isoelectric points that represent the protein is acidic nature, and it will help to purify the protein molecule. The extinction coefficient was 21680 at 280nm; it may possible to avoid interference of other substances. The evaluated value used to determine the quantificatin of protein – protein or protein-ligand interactions.

The quantitative measurement of dynamic equilibrium based on the half-life time. The TNF-α have 30 hours in mamalian reticulocytes, in *Yeast* have 20 hours and 10 hours in *E.coli*. The stability of protein was determined by using the instability index (40.75).

The aliphatic index represented that the volume of protein occupied by aliphatic chains (Alanine, Valine, Isoleucine and Leucine), TNF-α have 98.37 that denoted more stable in high thermal conditions. Grand Average Hydropathicity denoted that the hydrophobicity of aminoacid resiudes. Here TNF-α have -0.047 had a resonable interaction with water molecule. The protein molecule has 4 different atoms such as C, H, N, O and S, moleuclar formula was $C_{1152}H_{1825}N_{313}O_{337}S_{6.}$

3.2. Secondary Structure of Protein:

The secondary structure of TNF-α was predicted using SOPMA, SOPM and SSCP (Table 3). The protein was α helix with other structures such of extended stand, β turn and rantum coil (Table 4). It is shown to have 2 transmembrane helices of FLSLFSFLIVAGATTLFCLLHFG and the identified tranasmembrane region was visulaized by PROTTER (Figure 1) and Pepwheel EMBOSS (Figure 2).

3.3. Protein structure validation:

Ramachandran Plot

The predicted protein structure was validated by using the Ramachandran plot using PROCHECK software that shows the protein molecule contains 456 residues in that 206 aminoacid most favoured region, 99 aminoacid additionally allowed and 18 generally allowed and 10 aminoacids disallowed Table 6. The results are shown in figure 4. WHAT IF shows the ie Z-score of protein: -1.813

Pocket Finder:

The protein was tested by using the Pocket Finder, 10 numbers of pockets were indentified and the binding site had different binding volume. The datas were shown in Table 4.

3.4. Docking: Tumour necrosis factor, the Macromolecule and Ligands (isolated compounds of *Citrus reticulata* and Nitrofurazone) (Table 5a & 5b) were subjected to docking studies by using online Autodock software. The software used to runs 10 docking and were shown in Table 6, 7.

Amino acids	Numbers	Percentage
Ala (A)	19	8.2%
Arg (R)	14	6.0%
Asn (N)	7	3.0%
Asp (D)	7	3.0%
Cys (C)	4	1.7%
Gln (Q)	13	5.6%
Glu (E)	16	6.9%
Gly (G)	17	7.3%
His (H)	4	1.7%
Ile (I)	12	5.2%
Leu (L)	30	12.9%
Lys (K)	8	3.4%
Met (M)	2	0.9%
Phe (F)	10	4.3%
Pro (P)	15	6.4%
Ser (S)	20	8.6%
Thr (T)	10	4.3%
Trp (W)	2	0.9%
Tyr (Y)	7	3.0%
Val (V)	16	6.9%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%

Table: 1. Amino acid composition (%) of TNF α computed in protparam:

Accession No	Sequence Length	Mol. Wt	pI	-R	+R	EC	II	AI	GRAVY
P01375	233	25644.4	6.44	23	22	21680	40.75	98.37	-0.047

Table: 2. Mol.Wt- Molecular Weight; pI- Isoeectric Point; -R - number of negatively charged residues; +R - number of positively charged residues; EC - Extinction coefficient at 280nm; II - Instability Index; AI - Aliphatic Index; GRAVY - Grand average of Hydropathicity

Secondary structure	SOPMA	SOPM
Alpha helix (Hh)	67 is 28.76%	70 is 30.04%
3_{10} helix (Gg)	0 is 0.00%	0 is 0.00%
Pi helix (Ii)	0 is 0.00%	0 is 0.00%
Beta bridge (Bb)	0 is 0.00%	0 is 0.00%
Extended strand (Ee)	59 is 25.32%	58 is 24.89%
Beta turn (Tt)	14 is 6.01%	21 is 9.01%
Bend region (Ss)	0 is 0.00%	0 is 0.00%
Random coil (Cc)	93 is 39.91%	84 is 36.05%
Ambigous states (?)	0 is 0.00%	0 is 0.00%
Other states	0 is 0.00%	0 is 0.00%

Table: 3. RESULT of SOPMA and SOPM

S.No	Predicted site	Binding Box Around Selected Sites
1	Predicted site 1 Site Volume: 5835 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-36, 50, 12) Max Coords: (10, 94, 47)
2	Predicted site 2 Site Volume: 180 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-19, 66, -5) Max Coords: (-2, 77, 10)
3	Predicted site 3 Site Volume: 143 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-21, 67, 45) Max Coords: (-8, 83, 57)
4	Predicted site 4 Site Volume: 42 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-14, 44, 15) Max Coords: (-4, 55, 25)
5	Predicted site 5 Site Volume: 29 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-6, 49, 18) Max Coords: (4, 58, 28)
6	Predicted site 6 Site Volume: 29 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-12, 85, 11) Max Coords: (-1, 93, 20)
7	Predicted site 7 Site Volume: 27 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-15, 52, 35) Max Coords: (-5, 63, 44)
8	Predicted site 8 Site Volume: 29 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-7, 82, 33) Max Coords: (2, 93, 43)
9	Predicted site 9 Site Volume: 26 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-33, 53, 29) Max Coords: (-24, 63, 38)
10	Predicted site 10 Site Volume: 25 Cubic Angstroms Protein Volume: 51414 Cubic Angstroms	Min Coords: (-15, 63, 42) Max Coords: (-6, 74, 51)

Table: 4 Pocket Finders: TNF α

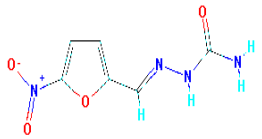
S.No	Name	Structure
1	Nitrofurazone	

Table: 5(a) : Standard Drug (Nitrofurazone)

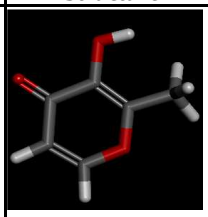
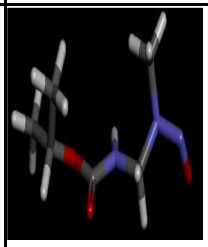
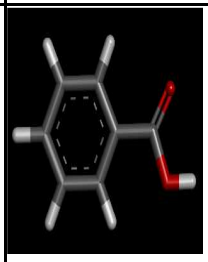
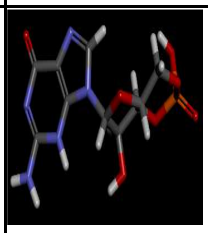
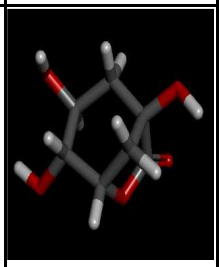
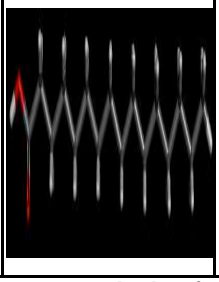
S. No	Name	Compound Structure	Activity
1	Maltol		Anticonvulsant, Antifatigue, Antioxidant, antitumour activities
2	Isopropyl methylnitrosamine		Antioxidant,antihyperglycemic.
3	Benzoic acid		Anesthetic;Antibacterial,Antiotitic,Antiseptic,Antiyeast,antifungal,Tyrosinase-Inhibitor.
4	GUANOSINE		Immunostimulatory activity Neuroprotective activity, Anticonvulsant
5	1,3,4,5 Tetrahydroxy cyclohexanecarboxylic		Antimutagenic, Antioxidant,
6	Palmitic acid		5-Alpha-Reductase-Inhibitor,Antialopecic, Antiandrogenic, Antifibrinolytic,Antioxidant,Hemolytic, Hypercholesterolemic.

Table: 5 (b): Plant Compounds Identified by GC-MS

Table: 6 Interacting residues responsible for Docking.

Docking Result	Est. Free Energy of Binding	Est. Inhibition Constant, Ki	vdW + Hbond + desolv Energy	Electrostatic Energy	Total Intermolec. Energy	Frequency (%)	Interact. Surface
Nitrofurazone (Drug) With TNF α	-3.72 kcal/mol	1.87 mM	-4.65 kcal/mol	+0.04 kcal/mol	-4.61 kcal/mol	20	391.356
1,3,4,5 Tetrahydroxycyclohexanecarboxylic with TNF α	-3.75 kcal/mol	1.79 mM	-4.26 kcal/mol	-0.06 kcal/mol	-4.32 kcal/mol	100	307.787
Benzoic acid with TNF α	-3.41 kcal/mol	3.15 mM	-3.69 kcal/mol	-0.02 kcal/mol	-3.71 kcal/mol	100	280.767
Isopropyl methylnitrosamine with TNF α	-2.67 kcal/mol	11.04 mM	-3.79 kcal/mol	-0.03 kcal/mol	-3.82 kcal/mol	10	383.58
Moltol with TNF α	-2.94 kcal/mol	6.98 mM	-2.95 kcal/mol	+0.01 kcal/mol	-2.94 kcal/mol	100	271.511
Palmitic acid with TNF α	-2.87 kcal/mol	7.91 mM	-6.29 kcal/mol	+0.01 kcal/mol	-6.28 kcal/mol	10	505.833
Quanosine with TNF α	-5.34 kcal/mol	122.27 uM	-5.26 kcal/mol	-0.11 kcal/mol	-5.37 kcal/mol	10	462.268

Table: 7 Docking Results

[IV] DISCUSSION

Wound repairing process is stimulated by interacting molecular signals and cytokines. The initial step is taken by cytokines which motivates and orchestrates the manifold cellular component of cytokine cascade. Macrophages play an important role in the healing process; formation of granulation tissue is one of the per-requirement in healing process. The macrophage product of

TNF-α performs potential action in wound healing.

The amino acid composition shows the presence of 30 Leucine residues and it is thought to play an important role in wound healing. The anabolic effect of Leu on protein metabolism at the whole body level (6,7,10) and at the level of some tissues such as muscle and liver (8,9,11) has been reported, its effect on protein metabolism in skin wounds [28].

The physiochemical parameters represent the protein primary properties. And then the secondary structure was predicted as alpha helical in nature of transmembrane protein in (Fig 1) and then the structure was evaluated by PROTTER and Ramachandran Plot represents the protein vaidation (Fig 2 & 3).

The TNF α and IL 6 stimulate the inflammatory process and reparative phase directly or indirectly. These factors enhance the endothelial and fibroblast functions directly, inducing cytokines and growth factors indirectly. TNF- α has 10 binding sites. Those binding sites have different site volumes but same protein volume (51414 cubic Angstroms) [29]. TNF-α one of the chemotactic factor for endothelial cells, and has a major role in capillary tube like structure formation in collagen gel [30]. The appropriate administration of TNF α can enhance the collagen amount during wound healing [31].

The docking results of TNF α with different plant compounds are shown in Fig 5. Table 9 shows the docking scores of these compounds with TNF- α. Among the result Quanosine had -5.34 kcal/mol free energy compare to that reference drug of nitrofurazone, The Quanosine seems to have a therapeutic potential for wound repair and skin production [32]. The healing process is not only enhanced by the Quanosine, but also 1,3,4,5, Tetra hydroxy cyclohexane carboxylic and Benzoic acid whose free energies are close to the standard drug.

The study therefore substantiates the fact that the compounds present in the plant extracts do cause wound healing. The docking results give a scientific understanding as to how these compounds work in the process of wound healing.

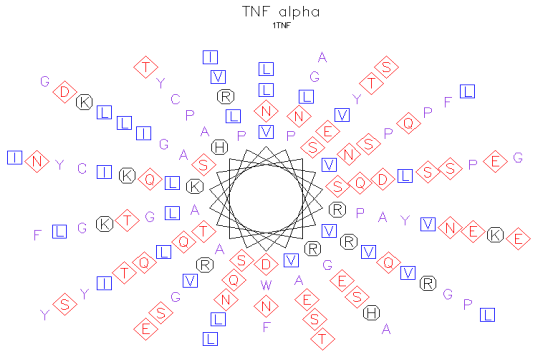


Fig: 1. Pepwheel of TNF α

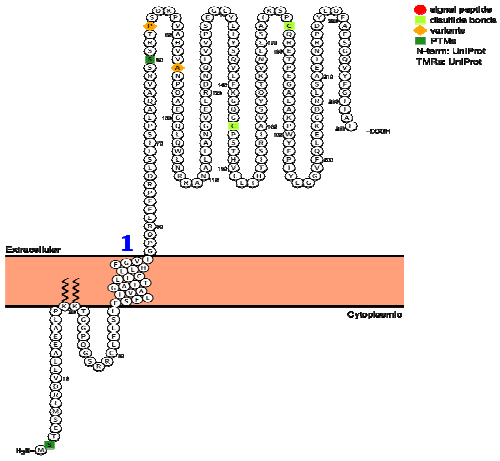


Fig: 2. PROTTER Result of Transmembrane region

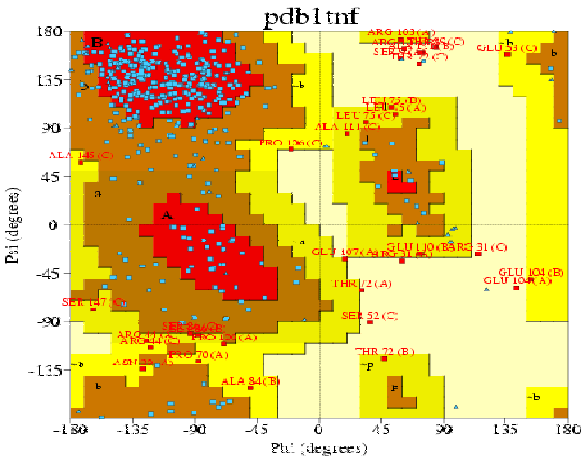


Fig: 3 Ramachandran plot

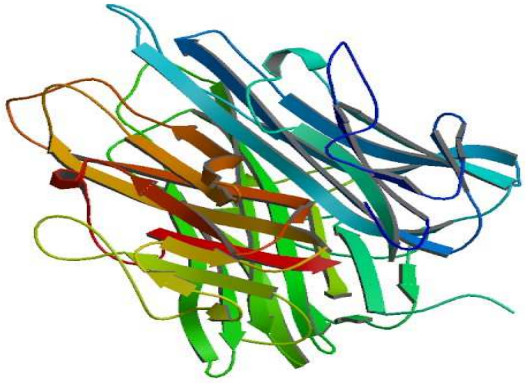
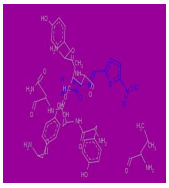
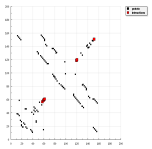
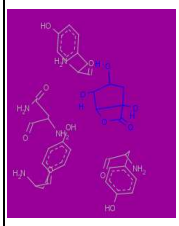
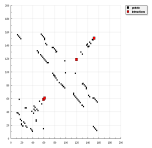
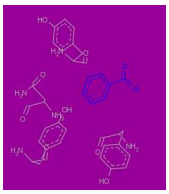
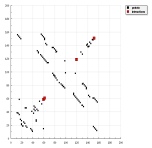
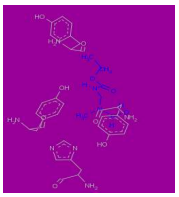
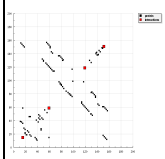


Fig: 4 Structure of TNF α: PDB ID: 1TNF

Docking of Compound with Protein Molecule	**Interactions**	**Hydrogen Bonding Plot**
Nitrofurazone (Drug) With TNF α		
1,3,4,5 Tetrahydroxycyclohexanecarboxylic with TNF α		
Benzoic acid with TNF α		
Isopropylmethylnitrosamine with TNF α		

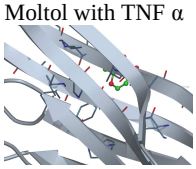
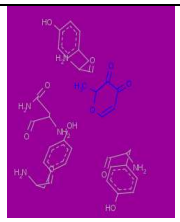
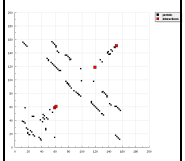
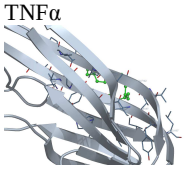
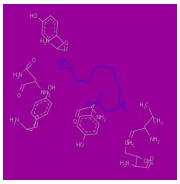
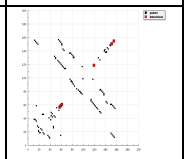
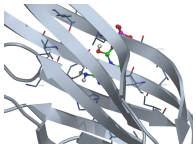
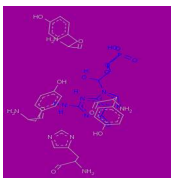
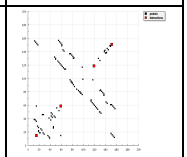
Moltol with TNF α 		
Palmitic acid with TNFα 		
Quanosine with TNF α 		

Fig: 5 Docking result

[V] CONCLUSION

TNF- α plays a vital role in wound healing, a detailed study of the physicochemical characteristics helps to understand its role in wound healing.

The physiochemical parameters also supported the protein properties, and then the protein undergoes with pockefinder to elucidate the sites whichwere ready to docking studies with ligand. In other hand the *Citrus reticulata* containing organic compounds were interact with the TNF-α protein and provide better scoring compare to that of standard drug of nitrofurazone by using Autodock. This study proves that Quanosine present in *Citrus reticulata* enhanced the wound healing process.

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