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RESEARCH PAPER

In silico analysis of heat shock proteins (HSPs) and their interaction with the bioactive molecules in groundnut (*Arachis hypogaea* L.)

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Abstract: Groundnut (*Arachis hypogaea* L.) is an important oilseed legume crop and its productivity is constrained by abiotic stresses, particularly drought and high temperature. Heat stress triggers the production of heat shock proteins (HSPs), which act as molecular chaperones to prevent protein misfolding, protein degradation, protein aggregation and maintain cellular homeostasis. Secondary metabolites such as flavonoids and phytosterols also play key roles in thermotolerance by functioning as antioxidants and membrane stabilizers. In this study, a total of 132 HSP sequences representing small HSPs (15), HSP70s (110), and HSP90s (7) were retrieved from the NCBI database and subjected to conserved domain and motif analysis. All proteins exhibited characteristic typical domains of their families, confirming evolutionary conservation. Phylogenetic analysis revealed gene duplication and diversification events, particularly within the HSP70 family. Representative sequences were modeled using trRosetta and refined through Ramachandran plot assessment. Molecular docking was carried out with selected ligands like flavonoids (flavanol, flavodione, hydroxyflavone, isoflavone, quercetin) and phytosterol (beta-sitosterol). Docking analyses revealed protein-ligand interactions, with beta-sitosterol showing the strongest binding affinity, with HSP90 (-10.05 kcal/mol) and HSP17 (-6.05 kcal/mol). These findings demonstrate a potential regulatory relationship between HSPs and secondary metabolites, highlighting beta-sitosterol as a promising secondary metabolite for enhancing thermotolerance in groundnut.

Key words: Groundnut, Heat shock proteins, Heat stress, Molecular docking, Secondary metabolites

Introduction

Groundnut (*Arachis hypogaea* L.) is an important legume crop belonging to the family Leguminosae and mostly cultivated in tropical and subtropical regions. Groundnut kernels are rich sources of edible oil (49.66%), protein (23.68%), carbohydrates (21.51%), fiber (8.0%), minerals, vitamins and antioxidants (resveratrol, flavonols, phenols and phytosterols) (Settaluri *et al.,* 2012). India is the second largest producer of groundnut, next to China, producing 10.29 million tonnes (Anon, 2023). The production of groundnut is vulnerable to several biotic and abiotic stresses during the crop growth period (Janila *et al*., 2016). Biotic stresses include diseases (rust, stem rot, leaf spots, *Aspergillus* infection, bud necrosis and bacterial wilt) and insect pests (cutworm, pod borer, leaf miner, thrips, and termites) (Salunke and Kenchanagoudar, 2021; Kona *et al*., 2024). Abiotic stresses include drought, high temperature, cold, salinity, and calcium-induced iron chlorosis (Chethana *et al*., 2020; Aravind *et al*., 2022). Among these, high temperature is a major factor limiting crop growth and yield.

High temperature in plants trigger the production of specialized proteins known as heat shock proteins (HSPs). HSPs act as molecular chaperones that prevent protein misfolding, aggregation and degradation, ensure proper folding, and maintain cellular homeostasis during stress (Nakamoto and Hiyama, 1999; Ellis, 2006). HSPs are classified based on molecular weight into HSP100, HSP90, HSP70, HSP60 and small HSPs (Vierling, 1991). In groundnut, small HSPs are rapidly induced under heat stress (>35°C) in heat-tolerant genotypes (Chakraborty *et al*., 2018) and qRT-PCR studies confirm upregulation of *HSP17* and *HSP90* in heat tolerant genotypes (Kokkanti *et al*., 2019; Aravind *et al*., 2024). Apart from HSPs, secondary metabolites such as flavonoids and phytosterols also play a crucial role in plant defense mechanism against abiotic stresses due to its antioxidant property and thus stabilizes the membranes and modulates the stress-responsive pathways. Among the phytosterols, beta-sitosterol, is a naturally occurring sterol molecule that is known to increase the activity of free-radical scavenging enzymes such as CAT (Catalase), SOD (Superoxide dismutase), POD (Peroxidase), APX (Ascorbate peroxidase) and in turn ensures the membrane stability under heat stress by decreasing the level of MDA (Malondialdehyde) (Rossi and Huang, 2022).

Previous studies have shown that, the genotypes with high antioxidant and polyphenolic content showed tolerance to high temperature tolerance (Gopal, 2023) and no direct interactions (*in silico*) were reported earlier between heat shock proteins and secondary metabolites. Considering the importance of flavonoids and phytosterols in abiotic stress defense mechanism, interaction studies with selected HSPs and secondary metabolites were conducted to understand the regulatory relationship between HSPs and bioactive compounds.

Material and methods

Database search and sequence analysis

Heat shock protein (HSP) sequences of *Arachis hypogaea* were retrieved from NCBI using keywords "HEAT SHOCK

PROTEIN17," "HEAT SHOCK PROTEIN70," and "HEAT SHOCK PROTEIN 90" and "AND *ARACHIS HYPOGAEA*". The sequences were examined for conserved domains, including the sHSP/α-crystallin family domain and ATPase N/C-terminal domains, using the ScanProsite tool of the ExPASy-PROSITE server (De Castro *et al*., 2006). Conserved motifs among full-length sequences were identified using the MEME 5.5.8 tool.

Multiple sequence alignment and phylogenetic analysis

Sequences were aligned using 'ClustalW' program in MEGA 12.0 with default parameters, gap opening penalty 10, gap extension penalty 0.10 and 0.20, respectively. Phylogenetic analysis was performed with the Maximum Likelihood method based on the Le_Gascuel_2008 model, with bootstrap consensus tree generated from 1000 replicates. A discrete Gamma distribution modeled evolutionary rate differences.

3D structure of protein and ligand

The 3D structures of HSPs were predicted using the trRosetta server, which generated five models for each protein in PDB format. The Ramachandran plot was analyzed in Swiss PDB Viewer, to identify disallowed regions, followed by loop building for model optimization. The structure of phytosterol (β-sitosterol) and flavonoids (flavanol, flavodione, hydroxyflavone, isoflavone and quercetin) with antioxidant properties were selected as ligands for the interaction.

Molecular docking

Protein-ligand docking was performed in AutoDock to predict binding conformation and interaction stability. Docking outcomes were evaluated based on hydrogen bond formation between HSPs and ligands, providing insights into their potential role in heat stress tolerance.

Results and discussion

Identification and domain analysis of HSPs in Groundnut

A total of 132 HSP sequences were retrieved from the NCBI database including small HSPs (HSP17), HSP70, and HSP90 families in *Arachis hypogaea*. The retrieved dataset included 15 small HSPs (sHSPs), 110 HSP70s and 7 HSP90s, which is an indicative of the expansion and diversification of HSP70 family genes in cultivated groundnut. Systematic genome-wide *in silico* identification and annotation of HSPs can be achieved through conserved domain analysis and motif analysis, sequence alignment and phylogenetic approaches. Similar approach were used by Guo *et al*., 2021; Razzaq *et al*., 2023.

Domain analysis using PROSITE scanner and MEME suite revealed that all proteins harboured conserved domains to their respective families. The sHSPs exhibited alpha-crystallin domains, essential for protein stabilization and refolding during stress (Fig. 1a, b). The HSP70s were characterized by N-terminal ATPase binding domains and substrate-binding domains (Fig. 1c, d), while HSP90s displayed N-terminal (N), middle (M) and C-terminal (C) domains associated with ATP hydrolysis and dimerization (Fig. 1e, f). The presences of these domains validate their classification and their role protein folding, prevention of aggregation, and cellular protection during heat stress. Similar domain conservation has been previously reported in other crops like wheat, soybean and rice (Kumar *et al*., 2020; Guo *et al*., 2021; Razzaq *et al*., 2023), confirming the evolutionary conservation of HSPs and underscoring their vital role in thermo tolerance mechanisms.

Phylogenetic analysis of HSP proteins

To assess the evolutionary relationships among the identified HSPs, multiple sequence alignment was performed using the Weighted Clustal algorithm, followed by dendrogram construction via the Neighbor-Joining (NJ) method in MEGA 12. The resulting phylogenetic tree clearly grouped the proteins based on their domain similarity: 15 alpha-crystallin domain-containing sHSPs, 110 HSP70s with ATPase/substrate-binding domains, and 7 HSP90s with N/M/C domains (Fig. 2). In order to check the correctness of phylogenetic analysis, an out group DREB2C (Dehydration-responsive element-binding protein 2C) is considered as a control sequence. HSP70, HSP90, and sHSP sequences are clustered into distinct groups based on sequence similarity, indicating their evolutionary divergence within the heat shock protein family. The analysis underscored the significance of evolutionary conservation and structural similarity among these proteins. The observed clustering patterns suggested gene duplication and diversification events, indicating an expansion of HSPs, particularly those involved in heat stress signaling. Alike evolutionary patterns have been reported in wheat (Kumar *et al*., 2020; Guo *et al*., 2021) and soybean (Razzaq *et al*., 2023). The secondary metabolites play

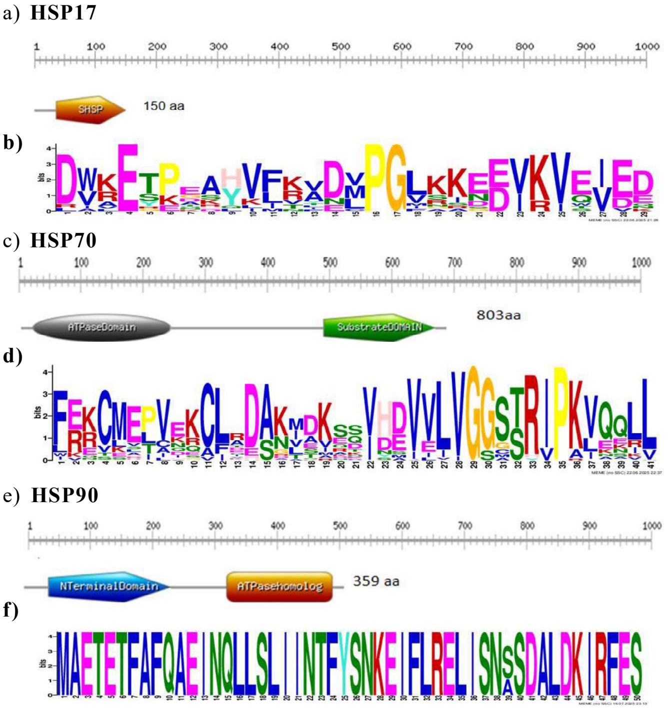


Fig. 1. Domains predicted using HSP protein sequences of *Arachis hypogaea* from NCBI: Alpha-crystallin domain (a and b); N-terminal ATPase binding domain or substrate binding domain (c and d); N/M/C dimerization domain (e and f) using PROSITE scanner and MEME

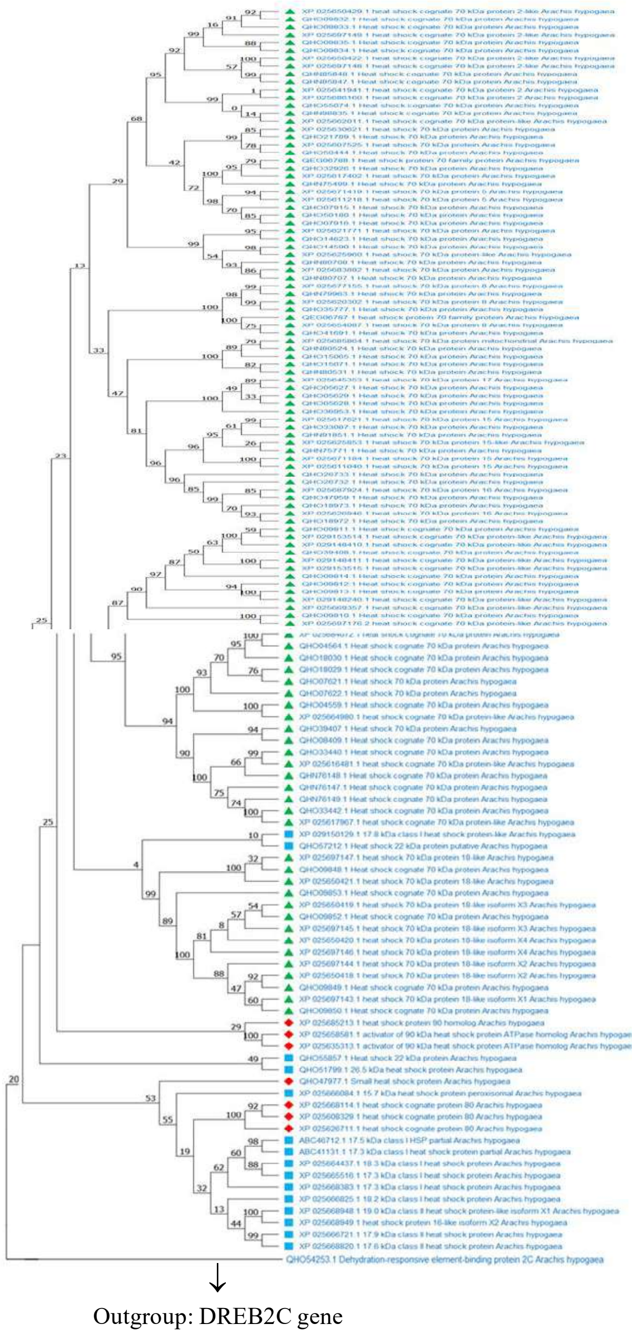


Fig. 2. Phylogenetic tree showing three groups of HSP protein families in *Arachis hypogaea*

a pivotal role in heat stress tolerance by maintaining ROS (reactive oxygen species) homeostasis, protecting cellular membranes, regulating cellular signaling, and activating genes and TFs (transcription factors) associated with metabolic pathways (Jan *et al*., 2021; Rehman *et al*., 2024).

Molecular structure prediction of proteins and metabolites

Due to the absence of experimentally resolved 3D structures for groundnut HSPs, representative sequences from each HSP family (based on phylogenetic similarity) were selected for computational 3D modeling using trRosetta. The tool generated five protein models in PDB format (Fig. 3a). Structural validation was carried out using Ramachandran plots, and loop building was done using SPDBV to shift the amino acids from disallowed region to allowed regions (Fig. 4). The finalized protein structures, with minimized steric hindrance and favourable conformations, were used in molecular docking studies with secondary metabolites. The SMILES format of the secondary metabolite was retrieved from PubChem and the 3D structure was predicted using the Corina server (Fig. 3b).

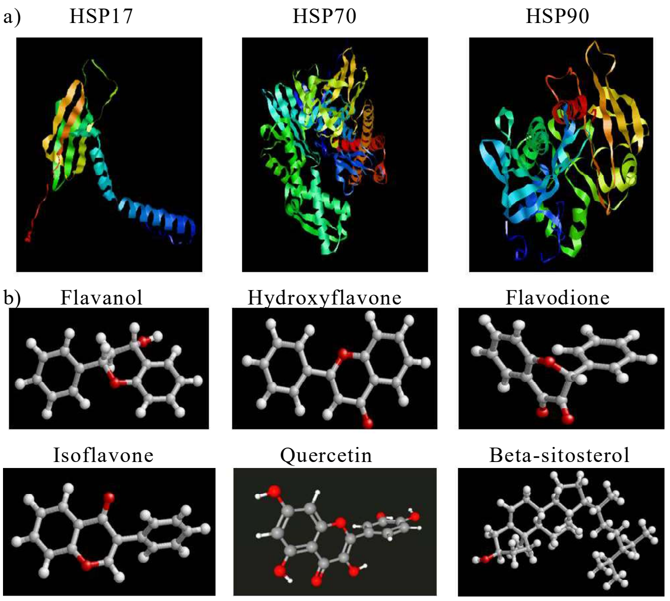


Fig.3. a) 3D structure of HSP17, HSP70, HSP90 b) 3D structure of ligands using Corina server

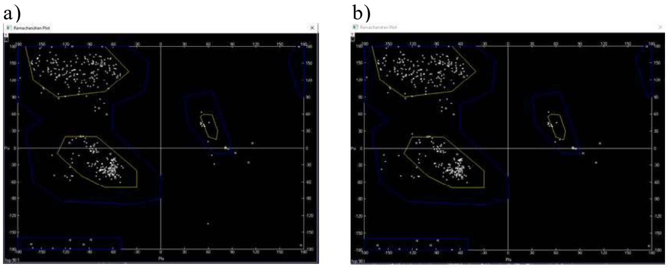


Fig. 4. Ramachandran plots a) Before loop building b) After loop building

Molecular docking with secondary metabolites

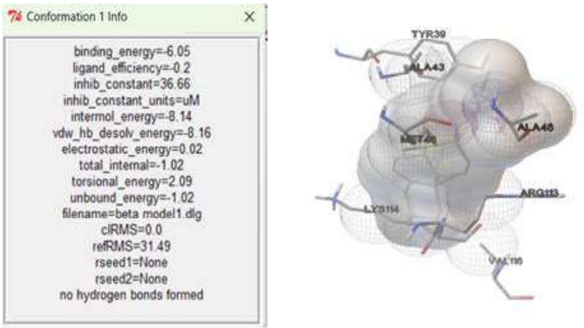
Following the identification and classification of heat shock proteins (HSPs), molecular docking studies were conducted to investigate their interactions with secondary metabolites. Previously, genotypes with higher antioxidant and polyphenolic content exhibit higher tolerance to high temperature (Gopal, 2023). In the present study, six secondary metabolite showcasing antioxidant acivity flavonoids (flavanol, flavodione, hydroxyflavone, is oflavone, quercetin) and phytosterol (β-sitosterol) were docked with HSP17, HSP70, and HSP90. A total of 18 docking interactions were analyzed, revealing that β-sitosterol consistently exhibited the strongest binding, particularly with HSP90 (-10.05 kcal/mol) and HSP17 (-6.05 kcal/mol) (Table 1, Fig. 5). Previous studies support these results, as quercetin enhanced salt and heat tolerance in rice by regulating *SOS* and *HSP16* genes (Jan *et al*., 2021), while β-sitosterol

Table 1. Molecular docking studies of heat shock proteins and secondary metabolites

Protein	Ligand	Lowest binding energy	Interactions
HSP17	Beta-sitosterol	-6.05	UNK:H67 LYS114:HZ2
HSP17	Flavanol	-5.38	UNK:H31 LYS114: OUNH1:O17ARG113:HE
HSP17	Flavodione	-5.22	UNK1:O9ARG113: HH22 UNK1:O11LYS114:HN
HSP17	Hydroxyflavone	-5.29	UNK1:H28 LYS114:HNUNK1: O10ARG113:HE 1
HSP17	Isoflavone	-4.04	UNK1:O9
HSP17	Quercetin	-5.93	ARG113:HH22 ARG113:HE LYS114: HN
HSP70	Beta-sitosterol	2.06	-
HSP70	Flavanol	-7.60	UNK1:H31 1THR126: O
HSP70	Flavodione	-8.12	UNK1:O18 1VAL75: O
HSP70	Isoflavone	-7.75	UNK:O9 17GLY77:HN
HSP70	Hydroxyflavone	-7.66	UNK1:H28 0
HSP70	Quercetin	-6.58	UNK1: H30 1
HSP 90	Beta-sitosterol	-10.05	ARG296 : HN
HSP90	Flavanol	0.60	-
HSP90	Flavodione	0.30	-
HSP90	Hydroxyflavone	0.60	-
HSP90	Isoflavone	-7.80	UNK:O9 1ASN93:OD 1
HSP90	Quercetin	0.60	-

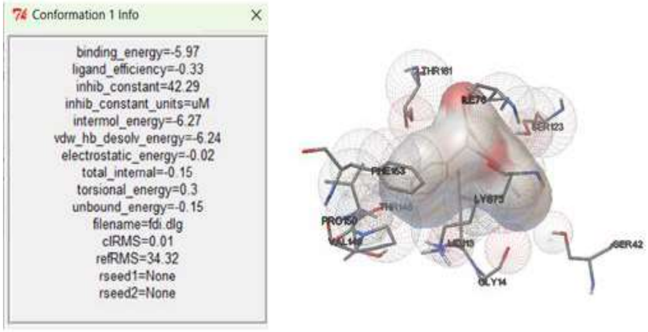
UNK/UNH- Unknown, H-Hydrogen, O-Oxygen, LYS-Lysine, ARG-Arginine, THR-Threonine, VAL-Valine, GLY-Glycine, ASN-Asparagine, HZ-Hydrogen atom of an NH group, HE-Hydrogen atom attached an epsilon-position atom of a side chain, HN-Amide hydrogen, HH-specific hydrogen from a side chain nitrogen, OD-Oxygen atom in the side chain of an aspartate.

HSP17 interact with beta-sitoserol



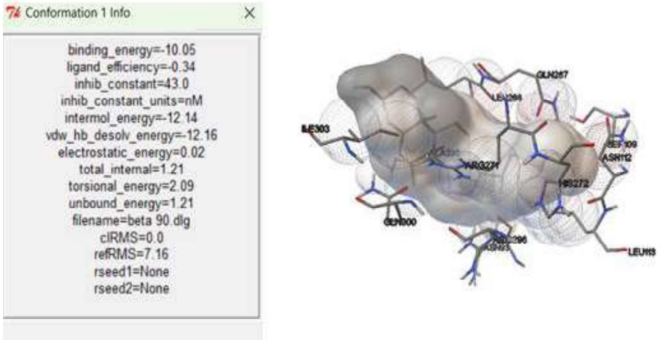
```
binding_energy=-6.05
ligand_efficiency=-0.2
inhib_constant=36.66
inhib_constant_units=uM
intermol_energy=-8.14
vdw_hb_desolv_energy=-8.16
electrostatic_energy=0.02
total_internal=-1.02
torsional_energy=2.09
unbound_energy=-1.02
filename=beta model1.dlg
clRMS=0.0
refRMS=31.49
rseed1=None
rseed2=None
no hydrogen bonds formed
```

HSP70 interact with flavodione



```
binding_energy=-5.97
ligand_efficiency=-0.33
inhib_constant=42.29
inhib_constant_units=uM
intermol_energy=-6.27
vdw_hb_desolv_energy=-6.24
electrostatic_energy=-0.02
total_internal=-0.15
torsional_energy=0.3
unbound_energy=-0.15
filename=fdi.dlg
clRMS=0.01
refRMS=34.32
rseed1=None
rseed2=None
```

HSP90 interact with beta-sitoserol



```
binding_energy=-10.05
ligand_efficiency=-0.34
inhib_constant=43.0
inhib_constant_units=nM
intermol_energy=-12.14
vdw_hb_desolv_energy=-12.16
electrostatic_energy=0.02
total_internal=1.21
torsional_energy=2.09
unbound_energy=1.21
filename=beta 90.dlg
clRMS=0.0
refRMS=7.16
rseed1=None
rseed2=None
```

Fig. 5. Interaction of HSPs with secondary metabolites using AutoDock

improved antioxidant enzyme activities and reduced lipid peroxidation under heat stress in bent grass (Rossi and Huang, 2022). A similar molecular docking conducted on HSP90 with dietary flavanols like Quercetin, Kaempferol, Myricetin showed the potential of flavanols as natural modulators influencing HSP90 activity and stability broadly showing anticancer effects in humans (Singh *et al*., 2015). In plants such studies on docking of HSP proteins with bioactive compounds have not been reported yet. This integrative approach highlights the functional relevance of HSPs - secondary metabolite interactions and identifies beta-sitosterol as a promising candidate for improving thermotolerance in groundnut.

Conclusion

This study provides a comprehensive *in silico* investigation of HSPs in *Arachis hypogaea*, encompassing identification, classification, structural modeling, and molecular interaction analysis. The data suggest that HSP17, HSP70, and HSP90 families harbour conserved domains and interact favourably with selected secondary metabolites, particularly beta-sitosterol, which may enhance heat tolerance by modulating HSP activity. This integrative approach reveals novel molecular insights into heat stress adaptation in groundnut and offers potential targets for crop improvement strategies under climate-resilient agriculture.

Acknowledgement

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