INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES

Volume-1, Issue-2 :2011 *www.ijpaes.com* *ISSN 2231-4490*

IN SILICO PREDICTIONS FOR MANIPULATIONS OF TESTIS SPECIFIC HEAT SHOCK PROTEIN HSP-70 GENES -HSPA2 AND HSPA1L WITH *CALOTROPIS* LATEX IN REGULATING MALE FERTILITY

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ABSTRACT: Regulation of male fertility is needed to protect the health of the society. It is well proved that Heat Shock Proteins (HSPs) influence various aspects of reproduction wherein the expression of HSP 70 is an essential part of sperm development. We reported the spermicidal effect of the latex of the medicinal plant *Calotropis*. In our effort to design dual action spermicide from *Calotropis,* in this study, different bioinformatics tools were used to find the possible manipulations of testis specific HSP genes-HSPA2 and HSPA1L with *Calotropis sp.* Besides the results of Ligand-Protein docking with Arguslab software, the predictions carried out online with STRING and STITCH databases along with GNCPro Gene Central Network and Array Express would be useful in custom designing of high throughput array experiments to have guided metabolite gene network in regulating male fertility with *Calotropis*- HSP.

Keywords: HSP-Heat shock proteins, HSF-Heat Shock Factor, *Calotropis* latex, Ligand Protein Docking, Gene interactions, Microarray expression

INTRODUCTION

The male reproductive health research needs have been identified as a research priority by the World Health Organization because the increase in the prevalence of male reproductive disorders during the past 50 years is evident in several epidemiological reports. The reproductive health status and behavior of men play a critical role in maintaining the health of the society and its progeny. In developing countries like India, due to lack of male involvement, sterilization is confined to females [1]. Under difficult socio-economic pressures, women in rural areas of India are resorting to different abortifacients and even feticides. But the problems arising due to male fertility is causing serious effects in all biosocial aspects including infertility, sexual violence and sexually transmitted diseases (STDs). The manipulation of male fertility is the most important and urgent need to prevent unwanted pregnancies and STDs. In order to formulate cost-effective health interventions aimed at men, the development of new fertility regulating drug from medicinal plants is an attractive proposition.Many indigenous plants are known to control birth, yet only few plants have been evaluated for their spermicidal activity. Our previous studies [2, 3] indicate the spermicidal effect of *Calotropis* latex and possible manipulations using Angiotensin Converting Enzyme (ACE) receptors of Renin Angiotensin System. In continuation of that, here we predicted the possible manipulation of *Calotropis* latex compounds through Heat-shock protein 70 in regulation of male fertility. HSP-70 is correlated with sperm maturity, function and fertility. Several HSP 70 family members are expressed in the mammalian testis and sperm [4]. In this study, the two testis-specific genes-(the isoforms of HSP70 chaperones) -HSPA2 and HSPA1L are used for prediction with different bioinformatics tools to evaluate the suitability of *Calotropis* latex compounds in regulating male fertility.

MATERIALS AND METHODS

Insilico Ligand-Protein Docking Studies: As reported in our earlier studies [2, 3], docking studies were done with Arguslab 4.0.1 software using the structures of the target genes - testis specific Human HSP 70 (HSPA2-3I33 and HSPA1L- 3GDQ) downloaded from Protein Data Bank [5]. The six small molecular latex compounds of *Calotropis* viz., Calactin, Calotoxin, Calotropin, Uscharin, Uscharidin and Uzarigenin were downloaded from PubChem. The binding sites were predicted using QSite Finder [6] by uploading the respective PDB file from Protein Database. Each docked molecule and different views of docked pose showing hydrogen bonds was viewed with Molegro Molecule viewer and the length of hydrogen bond was recorded as per the procedure reported [2,3]. To predict gene interactions, the online database STRING [7] was used. The interactions among a group of HSPA2 and HSPA1L genes were found out by text based entry in the search box tool. The interactions were represented graphically showing colored lines to describe interactions. The details of associated genes along with scores were tabulated with references. Similarly to find out the interacting compounds, gene names were entered in STITCH database [8] search tool box and the pictorial representations of the interactions along with references were recorded. The *in silico* research tool- GNCPro Navigator of GNC Pro Gene Network Central [9] was used to find the interactions for Heat Shock factor. In particular, the PCR Array Pathway (PAHS-076A) for Heat Shock Proteins given in the GNCPro Gene Network Central was used. Gene Array Express [10] the advanced computational tools allowed the online rendering of the selected testis specific HSP genes by extensively cross linking them to high-throughput microarray profiling. The microarray probe ids from Expression array for human, rat and mouse were compared for their expression at sample level, tissue level and at different stages of development. The details of reference probe ids suitable to our experimental protocol were recorded after comparison.

RESULTS AND DISCUSSION

Threats to men's fertility and reproductive health include infections, disease, cancer and exposure to heat stress and toxins. We have chosen HSP to find suitable manipulations for addressing all these problems. Testis specific HSP70 forms were first described in mice, but their homologues were later discovered in rat and human testis [11]. In humans, a number of genes are expressed in a testis-specific manner; yet we chose the differential expression of HSP 70 genes to get insight into the types of stress response as well as protection mechanisms. HSPA2 and HSPA1L have been selected in particular because studies have established human HSPA2 as an objective biochemical marker of sperm function and male fertility [12]. HSPA2 has been shown to be necessary for the progression of meiosis and is selectively expressed in mature spermatids and in sperm about to be released in the seminiferous tubuli [13]. The human HSPA1L is a cytosolic member of the Hsp70 family with high levels of expression in testis and spermatids and is not influenced by heat [13]. The HSPA2 protein was identified as a testis-specific creatinine kinase type M, the decreased activity of which has been connected with male infertility [14]. Different references in Pubmed show that *Calotropis* influences fertility, differently, both in male and female rats. Similarly, studies suggest that there are differences in the response to metabolism and blood pressure between males and females being fed with a high fructose. Hence it is necessary to study the effect of Testis specific HSP gene in our studies to differentiate the effect of *Calotropis* and/or Fructose feeding [2]. Here we used STRING and STITCH database to predict the interactions of HSPA2 and HSPA1L that could be possible *in vivo*. (Figure1 and 2, Table 1and 2). We made predictions for rat and mouse genes too. There were specific species differences between number of interactions and their relationships. For example, in Figure1.a, human HSPA2 had interactions with ten other related genes whereas in mouse, (Figure 1.b) Hspa2 had interactions with Hspa1b, Hspa1l, Hspa5 and Hsap8.

In rat (Figure 1.c) it was predicted to interact with Hspa1a, Hspa5, Hspa8 and Aft6. In both STRING and STITCH, the interacting genes were similar, except for the interacting chemicals. (Hence in Table-2, STITCH for HSPA1L shown, whereas STRING for HSPA1L not shown) The interacting chemical information for HSPA2 and HSPA1L were predicted with STITCH (Figure 2) wherein it is interesting to note that HSPA1L (Figure 2,Table2) had interaction with Abacavir the sulfate salt form of an agent that decreases Human Immunodeficiency (HIV) viral loads, retards or prevents the damage to the immune system, and reduces the risk of developing Acquired Immunodeficiency Syndrome(AIDS) [8].

Table -1 List of Predicted Interacting Genes with human HSPA2 in STRING

S..No	Predicted Functional Partners		Score
1.	NASP	Nuclear autoantigenic sperm protein (NASP);	0.905
2.	PARP1	Poly [ADP-ribose] polymerase 1 (EC 2.4.2.30)	0.836
3.	HSPBP1	Hsp70-binding protein 1 (HspBP1)	0.700
4.	CKM	Creatine kinase M-type (EC 2.7.3.2)	0.676
5.	MAP3K3	Mitogen-activated protein kinase kinase kinase 3	0.669
6.	TIMM44	Import inner membrane translocase subunit TIM44,	0.668
7.	MYBPH	Myosin-binding protein H (MyBP-H) (H-protein)	0.623
8.	GRPEL1	GrpE protein homolog 1, mitochondrial precursor	0.619
9.	TNNI1	Troponin I, slow skeletal muscle	0.613
10.	CDC2	Cell division control protein 2 homolog	0.611

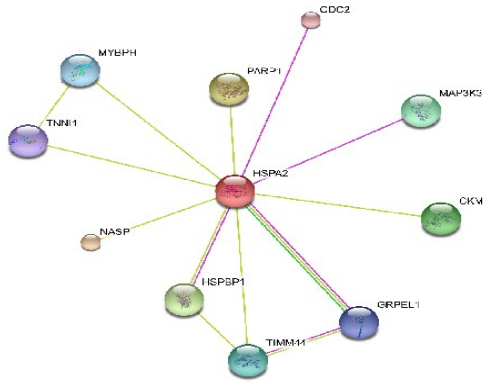

Figure 1.a :Gene interactions in human HSPA2 predicted with STRING

Protein-protein interactions are shown in blue, chemical-protein interactions in green and interactions between chemicals in red.
Chemical-chemical links are used to extend the network.

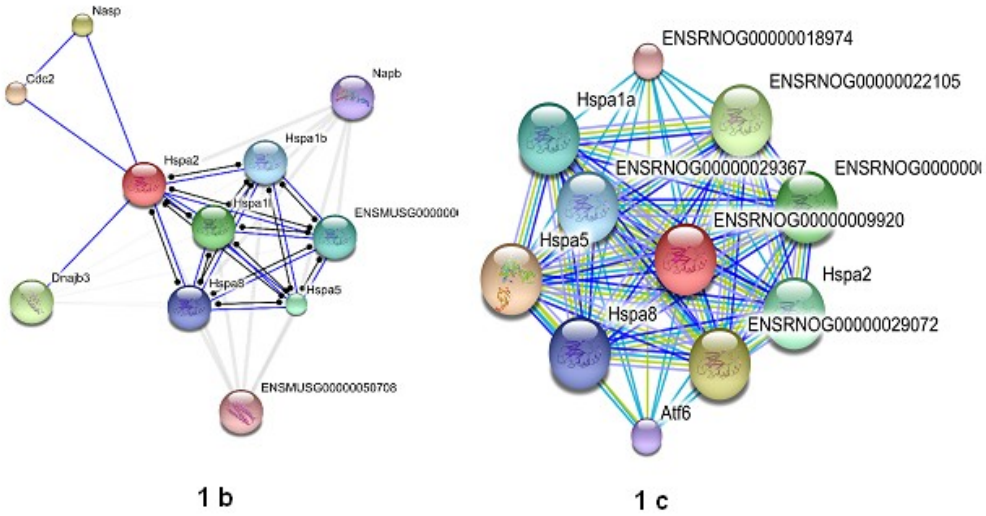

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Figure1.b: Gene interaction in Mouse Hspa2 predicted with STRING
Figure 1.c : Gene interaction in Rat Hspa2predicted with STRING

Protein-protein interactions are shown in blue, chemical-protein interactions in green and interactions between chemicals in red.
Chemical-chemical links are used to extend the network.

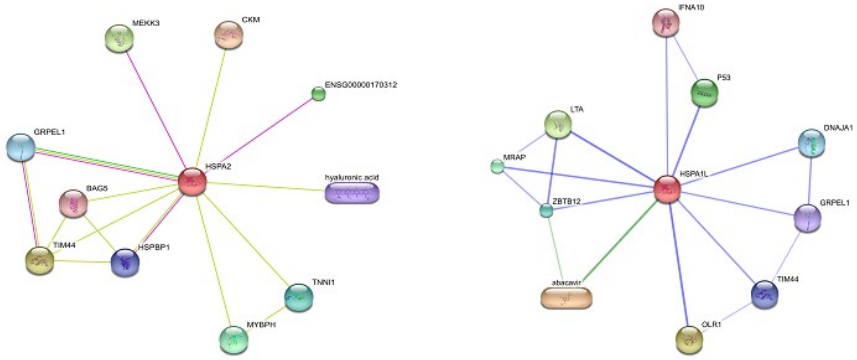

Figure – 2.Gene-Small Molecule interactions in human HSPA2 and HSPA1L predicted with STITCH

Protein-protein interactions are shown in blue, chemical-protein interactions in green and interactions between chemicals in red.

It is reported that Heat Shock Factor-1(HSF1) becomes activated in the testes in response to elevated temperature and protects spermatogonia viability; whereas it induces apoptosis of spermatocytes that are produced under adverse conditions, insuring that defective spermatozoa are not maintained [15].

Table -2 List of Predicted Interacting Genes-Small Molecule with human HSPA1L in STITCH

Sl.No	Predicted Functional Partners		Score
1.	abacavir	The sulfate salt form of abacavir	0.810
2.	OLR1	Oxidized low-density lipoprotein receptor 1 (Ox-LDL receptor 1)	0.778
3.	LTA	Lymphotoxin-alpha precursor (LT-alpha) (TNF-beta)	0.768
4.	P53	Cellular tumor antigen p53 (Tumor suppressor p53)	0.731
5.	MRAP	Melanocortin-2 receptor accessory protein	0.686
6.	ZBTB12	Zinc finger and BTB domain-containing protein 12	0.645
7.	DNAJA1	DnaJ homolog subfamily A member 1 (Heat shock 40 kDa protein 4)	0.640
8.	TIM44	Import inner membrane translocase subunit TIM44	0.610
9.	GRPEL1	GrpE protein homolog 1, mitochondrial precursor (Mt-GrpE#1)	0.588
10.	IFNA10	Interferon alpha-10 precursor (Interferon alpha-C)	0.583

The initial stimulus for activation of HSF-1 appears to be the exposure of hydrophobic domains of denatured proteins [16]. We used GNC Pro to predict the interactions of HSPA2 and HSPA1L with other HSP 70 genes, to examine the interaction of HSP 70 (HSPA) genes in human. The results summarized in both figure and tabular format are too large to be included here. Hence those results have been communicated separately. Using GNC Pro Navigator, the interactions of HSF1 were predicted. (Figure 3, Table 3). There were five up regulations, three down regulations, two Chemical modifications, eleven Physical interactions, two Predicted Protein interactions, eleven predicted Transcription Factor (TF) regulations and six other reactions. There were no co-expressions. Meiosis is critical for sexual reproduction, wherein HSP70-2 is required for desynapsis of synaptonemal complexes during meiotic prophase. Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility [17]. While Reverse-transcriptase-polymerase chain reaction (RT-PCR) analyses of gene expression confirmed this sex specificity, other study shows that relatively high levels of HSPA2 transcripts were found in various human non-testicular tissues [18].

Table 3- List of Genes added in Predictions for Heat Shock Factor-HSF1 Interactions

S.No	Gene	Ref Seq ID	Unigene ID	Description
1.	CEBPB	NM_005194	Hs.517106	CCAAT/enhancer binding protein (C/EBP), beta
2.	HMOX1	NM_002133	Hs.517581	Heme oxygenase (decycling) 1
3.	HSD17B6	NM_003725	Hs.524513	Hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)
4.	HSF1	NM_005526	Hs.530227	Heat shock transcription factor 1
5.	HSP90AA1	NM_001017963	Hs.525600	Heat shock protein 90kDa alpha (cytosolic), class A member 1
6.	HSP90B2P	N/A	N/A	N/A
7.	HSPA1A	NM_005345	Hs.520028	Heat shock 70kDa protein 1A
8.	HSPA4	NM_002154	Hs.90093	Heat shock 70kDa protein 4
9.	MAPK1	NM_002745	Hs.431850	Mitogen-activated protein kinase 1
10.	MAPK8	NM_002750	Hs.138211	Mitogen-activated protein kinase 8
11.	PLK1	NM_005030	Hs.592049	Polo-like kinase 1 (Drosophila)
12.	PRKDC	NM_006904	Hs.491682	Protein kinase, DNA-activated, catalytic polypeptide
13.	STUB1	NM_005861	Hs.592081	STIP1 homology and U-Box containing protein 1
14.	SUMO1	NM_003352	Hs.81424	SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae)
15.	YWHAE	NM_006761	Hs.513851	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide

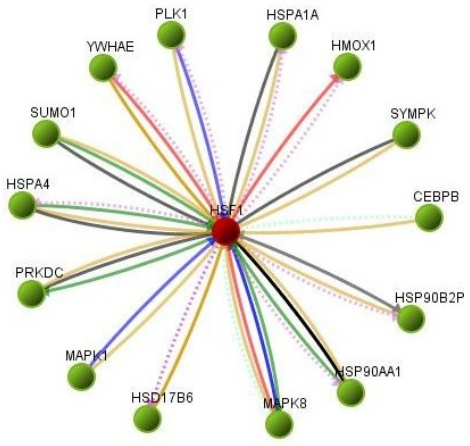

Figure-3 Heat Shock Factor (HSF1) Gene Interactions predicted with GNC Pro Navigator

Up-regulation Regulation Coexpression other Down-regulation
Chemical Modification Physical Interaction Predicted Protein Interaction

The use of cDNA microarrays for large-scale analysis of gene expression has proven to be useful in providing information about tissue and cellular function. In this study, Gene Array Express [10] was used for finding information on our candidate genes HSPA2 and HSPA1L. In Affymetrix human array expression, differences were seen clearly at normal tissue level and at different cancer tissue level. (Data and figures available online) We made predictions for rat and mouse arrays too wherein 2063 experiments were available with Affymetrix expression details on testis.(Data too large to be included). Further analysis provided useful information about the behavior of the selected genes during different stages of development. Coinciding with our predictions, studies reported that rats stressed during neonatal development showed changes in the expression pattern of Hsp70.Recent studies confirmed the extra-testicular expression pattern of mouse Hspa2; as they are regulated developmentally and expressed specifically in tissues[18], in this study too, the differential expression of genes may provide insight into which types of stress response mechanisms; The HSPA2 gene is the human homologue of the murine Hsp70-2 gene with 91.7% identity in the nucleotide coding sequence[19]. It is reported that the regulatory elements indispensable and sufficient for spermatocyte-specific activity of the rat HspA2 (Hst70) gene are localized within a 165 bp fragment between the T1 and T2 transcription start sites, encompassing exon 1 and the 5'part of the intron whereas the human HSPA2 gene is intronless and its transcription is initiated from a single start site [20]. With this prediction and literature details we selected 14 reference probe ids suitable to our experimental protocol in rats.

It is reported that the two crucial challenges faced by modern molecular Andrology are to find a solution for male infertility and to develop an effective, reversible male contraceptive [21].The frequency of testicular cancer and male infertility has been increasing in the past several decades [22]. The Testicular dysgenesis syndrome proposes an association between testicular cancer, male infertility, and genital abnormalities and its effect on the fetus [23]. It is demonstrated that lower HSPA2/CK ratios correlate with lower fertility rates in in vitro fertilization (IVF) programs [24]. The HSPA2 gene was down-regulated in sperm from infertile men and sperm HSPA2 activities are lower in men with varicocele suggesting that such anomalies of gene expression might be associated with pathogenesis in some subtypes of male infertility. [25] Besides HSPA2 polymorphism are reported in Alzheimer's, Systemic Lupus Erythematosus disease and in oxidative stress; HSP70-2+1267 genotype is a stronger predictor of septic shock in patients whereas HSPA1L gene polymorphism are associated with prostate cancer risk [26]. Hence to manipulate *Calotropis* latex compounds directed in HSP epitopes rendering for use in assisted reproduction and to design dual action microbicide from them, we did in silico Ligand Protein Docking studies. We selected two structures HSPA (3I33) and HSPA1L (3GDQ) [5] to develop drugs aimed at manipulation of human HSP. In this study, we removed the ADP molecules to know the exact binding sites because the ATP-dependent HSPA chaperone machine constantly shuttles between ATP-bound and ADP-bound states, which have different affinities for client proteins. In this study, the binding was predominantly with fifteen amino acids viz., Arginine (ARG), Alanine(ALA), Aspartic acid (ASP), Asparagine (ASN),Glycine(GLY),Glutamine(GLN), Glutamic acid (GLU), Histidine(HIS), IsoLeucine(ILE), Leucine (LEU), Lysine(LYS), Phenyl Alanine(PHE),Proline(PRO), Tyrosine (TYR) and Valine(VAL). (Figure 4 a.b, Table-4). With the receptor HSPA2 (3I33) - Calotoxin had six bonds. Uscharidin and Uscharin had five and four hydrogen bonds each whereas Calotropin and Calactin had three hydrogen bonds. In particular, the latex compounds had bonding with ARG, GLY and GLU in both receptors.

Table 4- Docking results of Calotropis latex compounds with Testis Specific HSP 70 genes

Calotropis latex compounds	HSPA2-3I33			HSPA1L- 3GDQ		
	Binding Energy	Binding residue	H Bond Length	Binding Energy	Binding residue	H Bond Length
Calactin	-9.84828	GLY 99(a) LYS 101(d) GLU 118(a)	2.27422 2.36205 2.59206	-9.79418	TYR43 (b) LYS 58 (d) GLU 233(a) ASN 237 (d) ARG 266 (d)	2.48925,3.50137 2.94625 3.56473 2.7238 3.00093
Calotoxin	-9.72792	VAL 60 (a) ALN 61 (a) ASN 238(d) ARG 267 (d)	2.90883 2.58985 2.49873,2.94175,3.2228 2.94405	-9.93652	ASN 176(d) PHE 219 (d) GLU 220 (a) VAL 221 (d) GLN 378 (d)	2.99902 2.9303 2.8995 2.679 2.60995
Calotropin	-9.92789	ARG 264 (d) ARG 267 (d)	2.01608,3.00168 2.67593	-10.9358	LYS 58 (d) LEU 91(a) PRO 93 (d)	2.39561 2.54053 3.41968
Uscharin	-10.1192	ARG 172 (d) ASN 175 (d) GLY 218 (a) VAL 222 (d)	2.85089,3.50363 3.01031 2.82582 3.56242	-10.0761	TYR 43 (b) LEU 91 (a) ARG 266 (d)	2.86898 2.8868 2.56869
Uscharidin	-9.77495	TYR 72 (b) HIS 90 (a) ARG 264 (d) GLU 271 (a)	2.57158 2.82292 2.99962 3.41863	-9.91101	ASP 188 (a) GLY 217 (a)	3.24186 3.27123
Uzarigenin	-10.8531	ALA 183(a) ILE 382 (a)	2.89979 2.50122	-9.59986	ARG 157 (d) ASN 176 (d) GLY 217 (a)	2.26701,3.08413,3.43459 2.7272 3.4972

***[Binding Amino acids status (a)- Acceptor; (d)- Donor; (b)- Both]**
Arginine (ARG), Alanine(ALA), Aspartic acid (ASP), Asparagine (ASN), Glycine(GLY) Glutamine(GLN) Glutamic acid (GLU), Histidine(HIS), IsoLeucine(ILE), Leucine (LEU), Lysine(LYS), Phenyl Alanine(PHE), Proline(PRO), Tyrosine (TYR) and Valine(VAL)

ALA was specifically bonded in HSPA2 whereas ASP was found in HSPA1L. The bonding with residues TYR 72, HIS 90, LYS 101 and ILE 382 was only once in HSPA2. For the receptor HSPA1L, Calactin, Calotropin and Uscharin had binding with the residues found in Site-1 and the residues bonded with Uscharidin and Uzarigenin are found in Site-7 of Q Site prediction. In the receptor HSPA2, the binding of Calotoxin and Uscharin with residues was predicted to occur in Site-3 as per QSite Finder results.The residues in particular TYR, ARG, ASN, GLU, GLY and LYS are found in both receptors. In this study too, we just report the binding sites and the prospective binding amino acid residue because the human homologue -HSPA2 additionally contains a six amino acid sequence near the carboxy terminal end not present in mice and rats [11]. The knowledge of binding sites and binding amino acid residues would be useful in designing wet lab experiments; In contrast to normal tissues, a significant expression of the HSPA2/HSPA1L protein was found in various human cancer cell lines and primary non small-cell lung cancer tissues. HSPA1L is a predictor for the prostate cancer risks [26] whereas HSPA2 has been considered important for cancer cell survival in ovarian cancer [27] it is reported that silencing of the HSPA2 gene led to growth arrest in cancer cells. We reported [2] the spermicidal effect on human sperm and identified binding amino acids with ACE proteins (ACE1 and testis specific-tACE) exactly correlating with that of a pharmaceutical used for treating tumors. This study also emphasizes that *Calotropis* compounds along with HSPs have a greater ability to activate the cell death-survival signals that regulate apoptosis during germ cell development. It is reported that oxidative stress and heat stress that would adversely affect sperm quality [28].

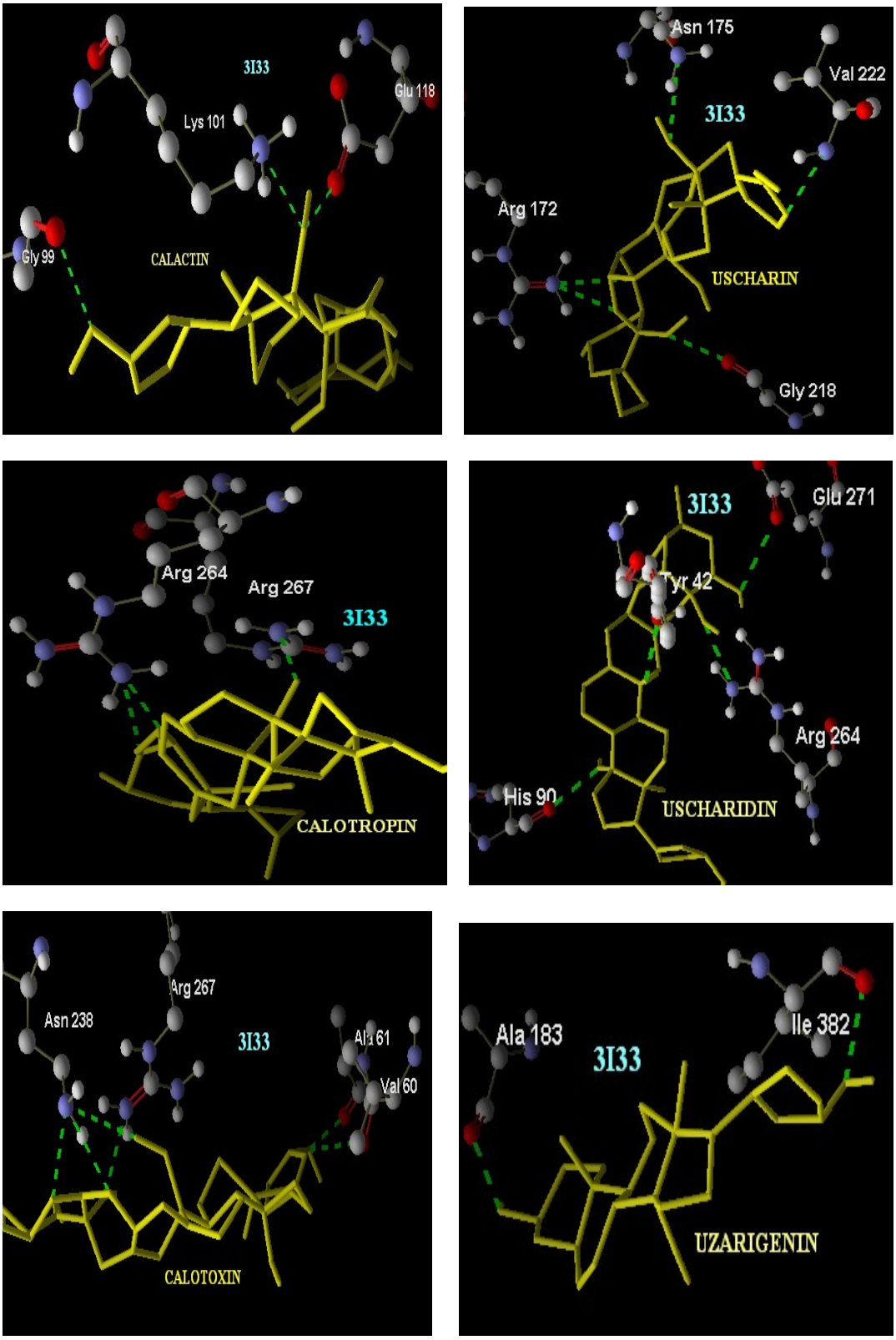

Figure-4.a-Docking results of *Calotropis* latex compounds with HSPA2 (3I33)

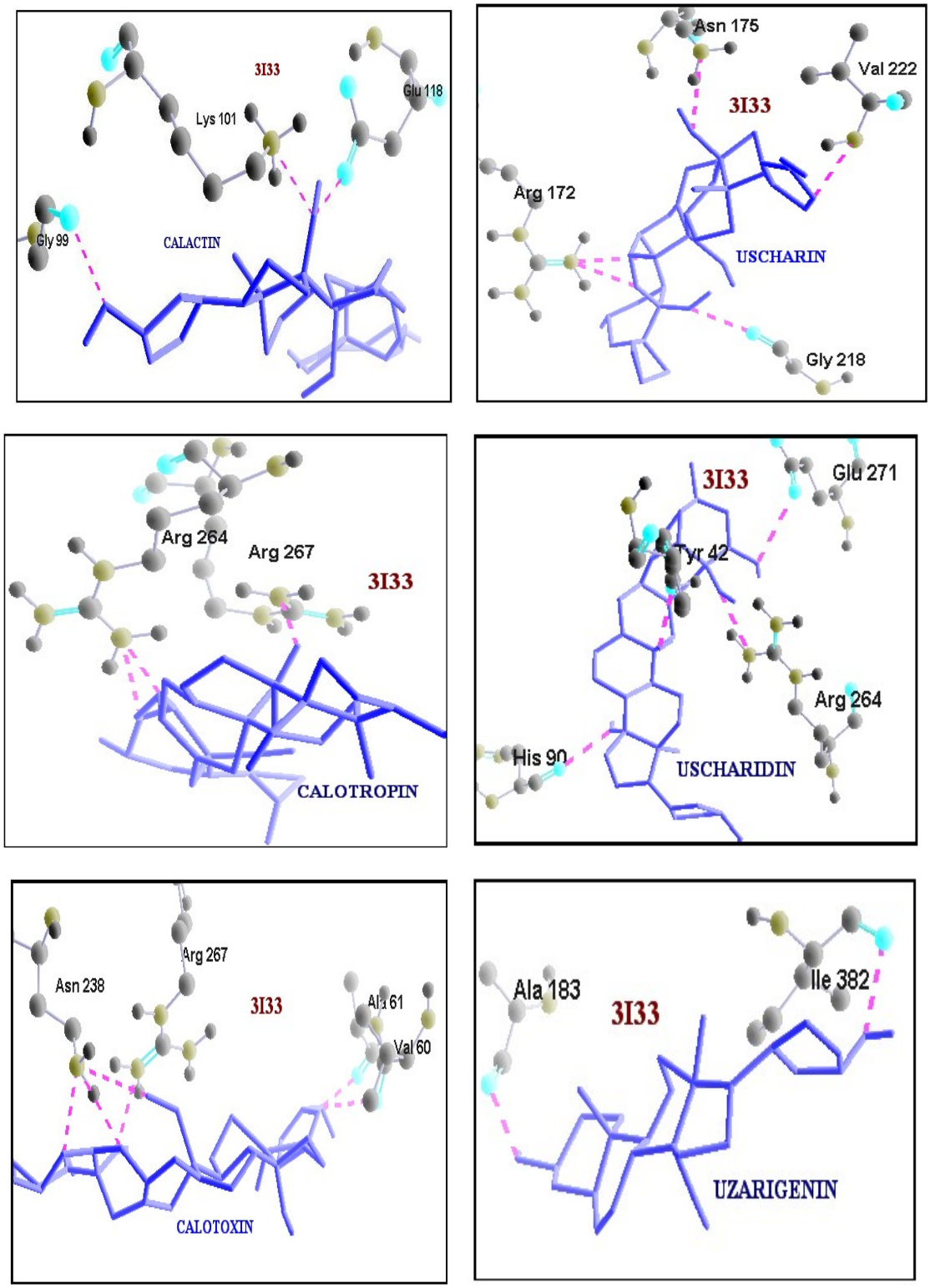

Figure-4.a-Docking results of *Calotropis* latex compounds with HSPA2 (3I33)
Ball & Stick- Receptor (3I33); Wireframe - Ligand (*Calotropis sp*); Dotted line- Hydrogen Bond

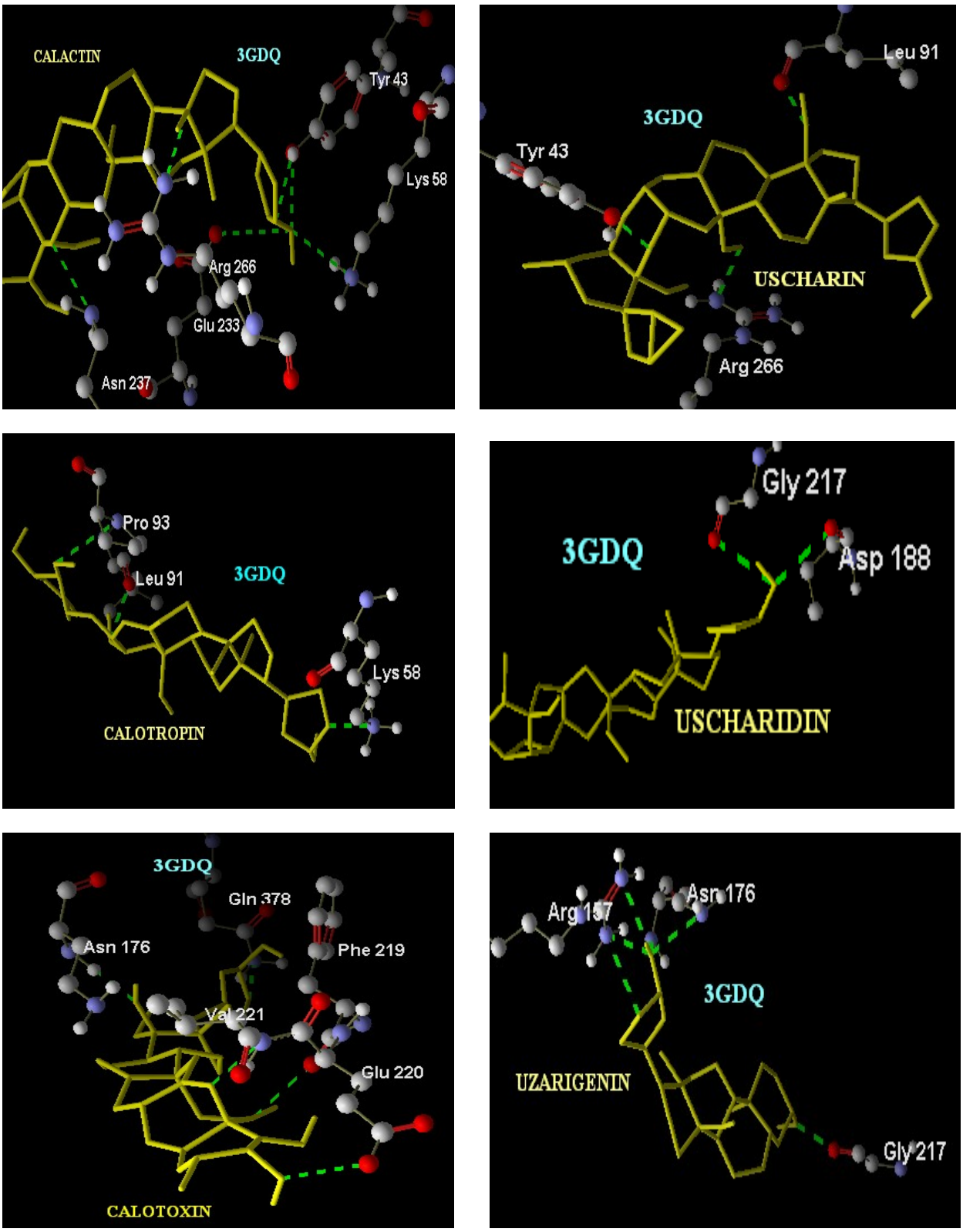

Figure-4.b-Docking results of *Calotropis* latex compounds with HSPA1L (3GDQ)

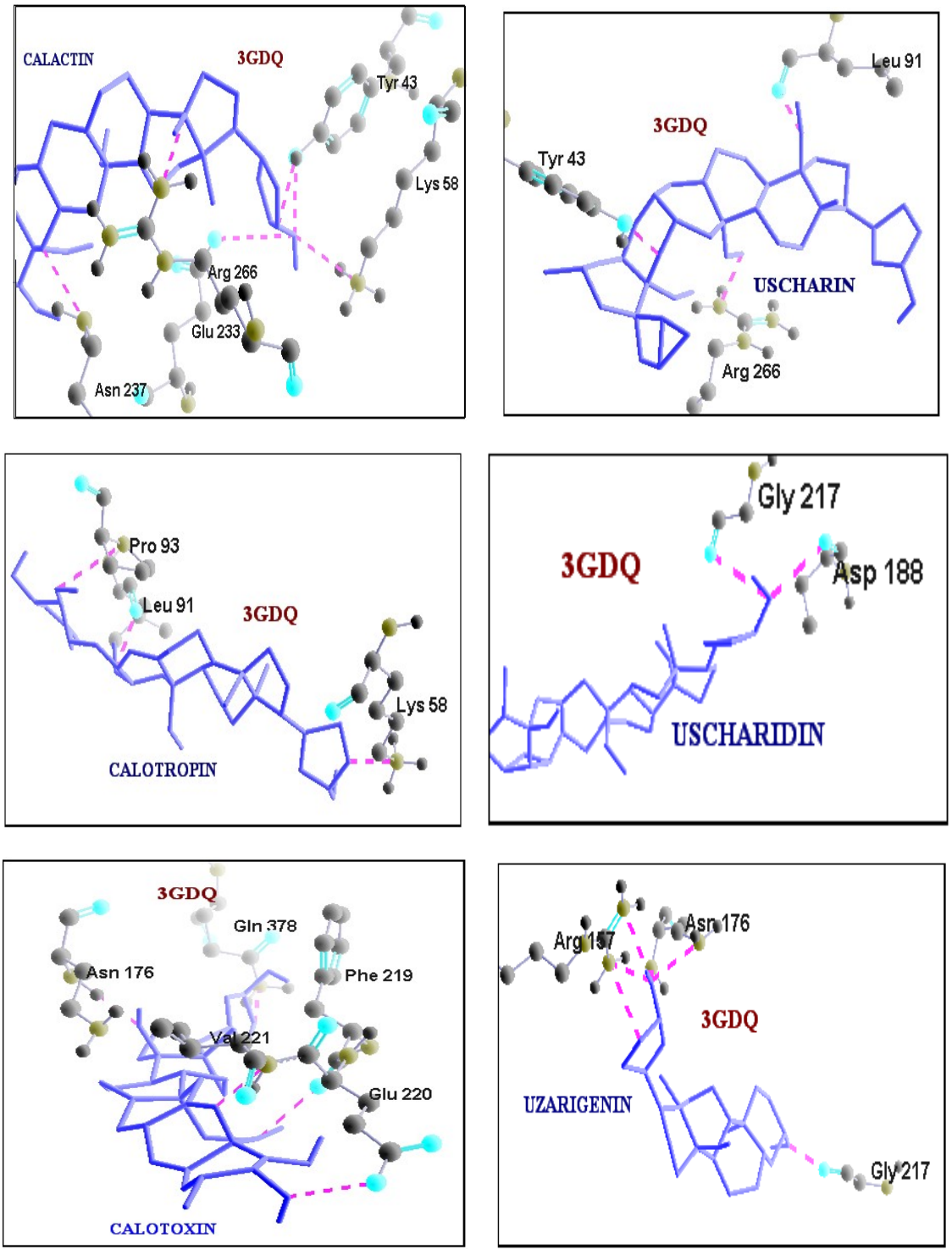

Figure-4.b-Docking results of *Calotropis* latex compounds with HSPA1L (3GDQ)
Ball & Stick- Receptor (3GDQ); Wireframe - Ligand (*Calotropis sp*); Dotted line- Hydrogen Bond

We have already communicated oxidative stress and antioxidant stress related metabolites that could be elicited in *Calotropis* and/or Fructose fed rats. The anti inflammatory activity of *Calotropis* is established in different experimental studies. Anti-inflammatory heat shock protein 70 genes are positively associated with human survival [29]. Though stressful manipulation of embryos in IVF culture enhances HSP expression, the results found in this docking experiment suggest that the effect of *Calotropis* compounds influencing HSP expression by predominant binding with amino acids ALA-GLU-ASP-GLY [30] could be targeted to enhance protection and survival of sperm after stress / exposure to IVF agents. The predictive studies made here helps in designing the high throughput array experiments. Yet autoimmunity and microbial pathogens, in particular -Chlamydia and Mycoplasma infection are the other confounding factors in male fertility regulation. As HSPs could detect microbial pathogens and autoimmunity, experiments are underway with *Calotropis*-HSP epitopes directed to detect immunity challenge including microbial HSPs.

CONCLUSION

By applying different Bioinformatics tools, this study has given details for the Ligand Protein docking and *in silico* prediction of HSP 70 gene (HSPA2 and HSPA1L) interaction network in identifying the candidate small molecules, genes network and micro array probe ids that need to be monitored in further high throughput experiments to evaluate the efficacy of Combined dose of *Calotropis* and Fructose in regulating male fertility.

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