Research Article

PREDICTIONS FOR HEAT SHOCK PROTEIN 70 RELATED GENE NETWORK AND METABOLITE CHANGES IN *CALOTROPIS* AND/OR FRUCTOSE FED RATS

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ABSTRACT

To better handle latex induced toxicity of the plant *Calotropis procera* (Ait). R.Br, it is necessary to understand the network of interactions and metabolite generation that occur in the cell. Heat Shock Proteins (HSPs) are key components contributing to cellular homeostasis in cells both under optimal and adverse growth conditions. HSP70- the highly conserved, abundant molecular chaperones are found in every species and in nearly every cellular compartment in eukaryotes. The changes elicited by *Calotropis* latex in HSP 70 is not reported so far. Our previous reports described the metabolite changes elicited in plasma of *Calotropis* and /or Fructose fed rats. In this study, we predicted the changes in HSP related gene network and plasma metabolites profiles by the web based data mining and Gene Network prediction methods using different bioinformatics tools. The *in silico* predictions were carried out with online MassTRIX, PolySearch, Rat Genome Database, Small Molecule Pathway Database and GNC Pro Gene Central Network and Genevestigator. The predictions revealed the candidate metabolite markers and associated gene network for the HSP defense giving new insight in the physiological states of fructose and /or *Calotropis* induced changes in homeostasis besides giving clues for designing custom arrays for the use of *Calotropis* in drug discovery.

Keywords: HSP-Heat Shock protein, Metabolite markers, Gene interactions, Data mining, Gene Network Predictions, Gene expression arrays.

INTRODUCTION

The ability of any organism to survive and adapt to severe systemic physiological stress is critically dependent on the appropriate compensatory stress response. HSP70 -the most important protein in HSP family generates a protective effect against injuries in the presence of various stresses and metabolic insults including the proteolytic toxicities. One such proteolytic toxicity where the remedy is undefined- is the accidental exposure of toxic weed *Calotropis* sp causing irritation of eye, skin and other related complications. Though the plant is well known for traditional medicinal uses, different news and reports reveal the fact that the plant latex is purposefully misused as foeticide and abortifacient.[1] We are undertaking a wide range of studies to elucidate the methods for handling *Calotropis* latex induced toxicity. We reported the use of both *in silico* and *in vivo* methods[2,3] to study the metabolic effect of *Calotropis* latex on Renin Angiotensin System. Studying the effect of *Calotropis* latex on HSP 70 is required because this evolutionarily conserved defense mechanism needs to be cross validated in different species, for practical manipulations in drug discovery. Since their initial identification and characterization, the stress proteins - HSPs have fascinated biologists and hence the scientific reports on them are abounding; even there is a separate database for the Molecular chaperones- HSP 70.[4] This study aims to highlight the significance different classes of metabolites and genes of HSP 70 interactions in understanding the stress induced responses in *Calotropis* and/or Fructose fed rats using different *in silico* tools, online databases and open access LCMS post data analysis softwares.

MATERIALS AND METHODS

Our previous reports[2,3] describe the details of plant extract preparation and the Animal studies (IAEC.No. Biochem BWC.002/2009). [Group 1- Control rats (Six); Group II- Six rats orally administered with aqueous extract of dried latex (DL) of *C. procera* at 300mg/kg body weight for a period of 15 days; Group III- Six rats fed with 10% fructose in drinking water for 15 days; Group IV- six rats dosed with DL of *C. procera* at 300mg/kg through oral gavage along with 10% fructose in drinking water for 15 days]. We have described[2,3] The LC-MS/MS analysis performed for studying the first pass effect of plasma on a Waters Alliance 2695 HPLC pump coupled to an electrospray (ESI) triple quadrupole Quattro Ultima mass spectrometer. The Instrument control and data acquisition were performed in scan mode (range 100-2000 m/z) of both positive and negative ions for 10 minutes using the MassLynx™ V 4.1 software. Our report contains details of the post data processing for differential analysis, spectral filtering, peak detection, alignment and normalization using Mass Lynx 4.1, and MZmine 2.0 softwares, besides custom search with NIST (National Institute of Standards and Technology) and search HMDB (Human Metabolome Database) databases.

In this study, peak identification was done using MassTRIX[5] and the Small Molecule Pathway Database (SMPDB).[6] The ionization mode (positive or negative) and ionization adduct was given as user selected parameters.

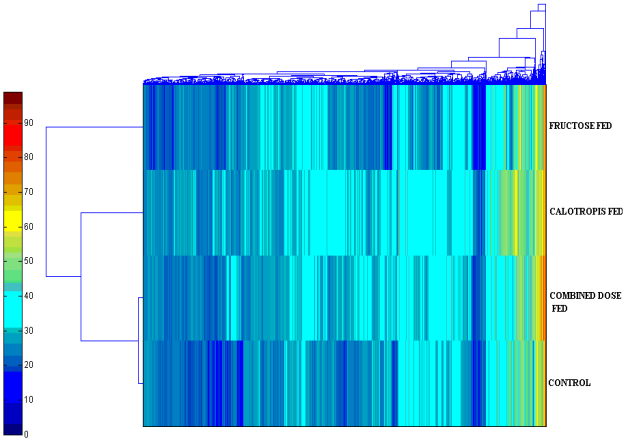
The isotopic pattern similarity was used as a second filter to select optimal candidates, by comparing the ratios of the matching peaks from the predicted isotopic pattern of the database compound. Database contents and related information were retrieved through a text- based search engine in Rat Gene Database.[7] PolySearch,[8] the web-based text mining system was used to collect information on metabolite to gene information and plausible "hits" were typically summarized in a tabular format. GNCPro Navigator,[9] free online software with *in silico* research tool was used for collating gene and pathway interactions. In particular, the PCR Array Pathway (PAHS-076A) for Heat Shock Proteins integrated in the GNCPro Gene Central Network was used. The interactions among a group of genes were represented graphically and the results generated had colored lines describing interactions, the details of which were tabulated giving biological discoveries derived from a collection of PubMed abstracts. The advanced computational tools in Genevestigator[10] allowed the online rendering of all interactive genes by extensively cross linking them high-throughput proteomic screening and microarray profiling. The chemical composition and disease association data were collected from Comparative Toxicogenomics Database (CTD)[11] and chemical data repositories to provide comprehensive information for each metabolite-gene in the pathway.

RESULTS AND DISCUSSION

We are undertaking a series of experiments to highlight the potential of *Calotropis* plant. It is well known that *Calotropis* plant survives extreme environmental stress including changes in temperature, the presence of free oxygen radical, heavy metals, pests or disease. Yet the proteolytic toxicity of *Calotropis* plant on human requires effective handling techniques. The HSP70 family represents one of the largest stress protein families with related members distributed throughout various intracellular compartments. Heat shock proteins protecting cells from a range of stresses is highly applied in many clinical applications of human diseases.[12] Hence, in this study the predictions for HSP related metabolite and gene network changes are chosen to delineate the difference that *Calotropis* compounds could be tailored to produce *in vivo*. For minimizing animal usage, we are highlighting the availability of different bioinformatics tools and data analysis softwares[2,3] that could be used to study the specific group of candidate metabolites/biomarkers for hypertension, oxidative stress, lipid metabolism and so on to handle *Calotropis* latex induced toxicity. Acute activation of the stress –HSP response modulates many aspects of physiology and drugs modulating HSPs are already in clinical trials. Though different members are available in HSP family, the discovery of small molecules altering Hsp70 expression and function is critical [13] because there are important differences in the biochemical pathways activated by different stressors and/or small molecules. In this study we attempted to use *in silico* tools and data mining softwares because focused interaction of HSP 70 related metabolite and gene network in manipulating *Calotropis* toxicity could be done only *in silico*. It is important to emphasize that with Quantitative Structure Activity Relationship (QSAR) studies, targeting the selective down regulation of Hsp70 to induce massive caspase-independent tumor cell death in lung cancer,[14] UNBS1244 - a novel cardenolide identified in African specimens of *C. procera* was used as templates for synthetic modification of UNBS1450[15] to produce anticancer drug. Targeted induction of HSPs for the prevention of insulin resistance is reported in high fructose fed rats; [16] Increased HSP70 and decreased COMT genes expression were observed, underlying the hypertensive effect of dietary fructose.[17] Different reports in Pubmed describe that over expression of Hsp70 reduced ischemic injury. As *Calotropis* latex contains many proteolytic enzymes, stress interactions generated *in vivo* have to be analyzed carefully to identify the metabolite changes exclusively elicited by *Calotropis* from that of HSP. The catalog of many companies contain a diverse panel of immunological and biochemical reagents specific for studying the different biochemical and immunological reactions of HSP70chaperones. This "same old" attitude has to change if new personalized medicine /molecularly based drugs are to be developed and that the patients could benefit from them. It is proved that the Metabolite profiling is desirable even in surgical suite as these methods provide highly specific and sensitive results to facilitate instant analysis of metabolites in cell lysates, serum, plasma and tissue extracts.[18]

Our previous reports highlight the metabolites elicited in all the four groups of rats using NIST and HMDB. As small molecule plasma metabolite resources in rat are limited, we used MassTRIX to convert the mass values into metabolites in KEGG rat pathway.[19] The data analysis with traditional multivariate technique is given as Biomarker Cluster map (Figure 1).

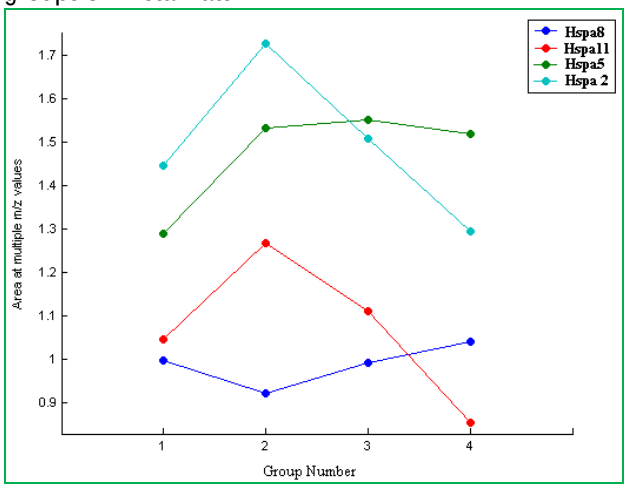
Figure 1: Biomarker Cluster Map for HSP 70 genes related metabolites in Wistar rats.



Biomarker Cluster Analysis for HSP 70 related metabolites in all four groups of rats. At the top, Clusters show grouping of peaks for metabolites /their intermediates. On left side, clusters of groups showing difference between treatment groups. Color scale shows intensity of peaks.

The Principal Component Analysis (PCA) and Clustering algorithm revealed that all the three treatments showed differences in the elicited HSP70 related metabolite profiles (Figure 2).

Figure 2: HSP 70 genes related metabolite changes in four groups of Wistar rats.



Group-1: Control; Group-2: *Calotropis* fed; Group-3: Fructose fed; Group-4: Combined Dose fed rats.

While the direct application of existing chemical name recognition tools revealed narrower focus of the metabolites, considerable manual efforts in mining different databases helped in identifying metabolite information relevant to HSP 70. Besides experiments reported into metabolite databases such as HMDB and SMPDB, the guidelines for nomenclature of HSP 70[20] was used in conjunction with the scientific literature in RGD for the identification of gene names and metabolite names along with text mining in CTD. [11] In this study, Metabolite name identification solved only one part of the problem to understand the stress response to toxicity/ disease; The integrated analysis of metabolite changes and transcriptomics of genes is required to understand toxicity or pathogenesis of diseases. In particular, the polymorphism of HSP 70 genes is believed to be involved in various diseases.[21] The contribution of the five polymorphisms in three genes (HSPA1A, HSPA1B, and HSPA1L) of Hsp70 family to essential hypertension is reported. Here we selected all the ten genes of Hsp 70 relevant to rat as given in RGD. Table 1 shows the details of the selected ten genes of Hsp70 present in rat with their IDs besides relevant description of their pathway, disease association and the number of the relevant metabolites identified in this study. Figure 3 shows the selected HSP genes interacting with each other and the neighbors using the GNC Pro Navigator tool leading to robust and well annotated reaction networks. Table 3 shows the description of HSP genes and their interactions (Figure 3) with reference in literature. The rationale for studying this interaction is that, genes coherently expressed in a large set of hybridization, sharing a common regulator may be influencing each other or may be involved in the same pathway. In this study, Hspa2, Hspa4l and Hspa14 did not have any interaction in the first run of GNC Pro Navigator. In particular, by multiple runs of GNC Pro and by adding neighbor genes some interactions were evident, suggesting that they could be possible. Studies show that the protective HSP70 effect could be due to the removal of toxic proteins and protein aggregates.[21-23] Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress.[24]

In this study, it is interesting to note that three members (Hspa1a, Hspa4 and Hspa8) of this Hsp 70 family had interactions with HSF1. (Given in Figure 3 and Table 2). We also added the results of the HSF1 interactions, by the lookup with GNC Pro to extract new potential information about the correlation between genes in Hsps and HSF; moreover we ran the program for all the Hsp 70 genes separately, and produced evidence of gene clustering in many cases. (Data too large to be included)

Table 1: Data mining for HSP 70 genes in Rat

HSP 70 genes	Description	No.of Metabolites identified in this study*	RGD ID	Rat Chromo some. Number	CTD		No. of References	
					Pathway	Diseases	RGD	Pubmed
Hspa1a	inducible protein 70 heat shock	9	1593284	20	8	434	2	9
Hspa1b	heat inducible gene; induced by global ischemia and kainic acid-induced seizures	3	2840	20	7	307	22	44
Hspa1l	heat shock protein 1-like	1	1595925	20	6	164	1	6
Hspa2	heat shock protein alpha 2 Testis-specific heat shock protein-related gene hst70	3	620664	6	6	141	6	3
Hspa4	heat shock protein 4 Estrogen signaling pathway	7	628878	10	-	-	7	53
Hspa4l	heat shock protein 4 likeosmotic stress protein 94 kDa (predicted);	0	1306528	2	1	138	1	3
Hspa5	heat shock protein 5; 78 kDa glucose-regulated protein	10	2843	3	6	435	9	23
Hspa8	expressed in unstressed cells	29	621725	8	9	389	12	29
Hspa9	heat shock protein 9	7	1311806	18	1	259	4	5
Hspa14	heat shock protein 14	0	1303296	14	None	17	3	1

Table 2: HMDB IDs of Metabolites identified for Genes Hspa5 and Hspa8 using PolySearch

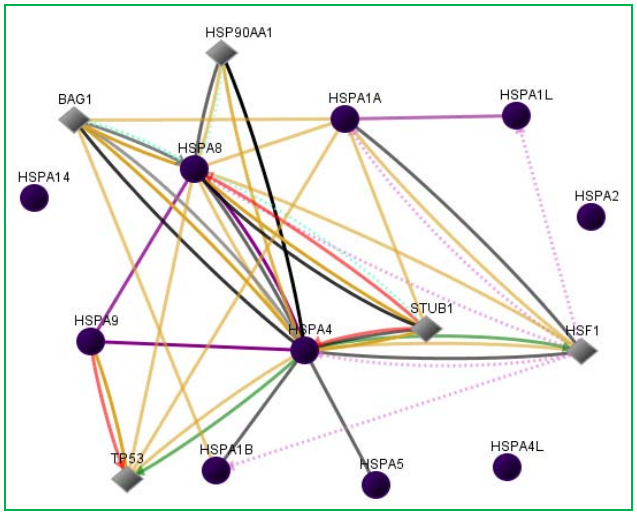
Sl. No.	Plasma Metabolite IDs			
	m/z	HSPA5	m/z	HSPA8
1.	125.014664	HMDB00251	103.063332	HMDB00112
2.	144.115036	HMDB01877	121.019753	HMDB00574
3.	146.118103	HMDB00895	131.094635	HMDB00687
4.	147.053162	HMDB02393	132.053497	HMDB00168
5.	167.094635	HMDB02182	146.069138	HMDB00641
6.	179.079376	HMDB01514	164.083725	HMDB00543)
7.	256.240234	HMDB00220	296.177643	HMDB01926
8.	318.064056	HMDB04068	302.042664	HMDB05794
9.	418.271912	HMDB05007	416.329041	HMDB00430

Table 3: Interactions of HSP 70 genes predicted with GNC Pro Navigator

Interactor 1	Interactor 2	Type	Pubmed ID
BAG1	HSPA1A	Physical Interaction	11101523,11741305, 9305631
BAG1	HSPA1B	Physical Interaction	11231577
HSF1	HSPA1B	Predicted TFactor Regulation	
HSF1	HSPA1L	Predicted TFactor Regulation	
HSPA1A	HSF1	Physical Interaction	17873279
HSPA1A	HSF1	Other	9699716
HSPA1A	HSF1	Predicted TFactor Regulation	
HSPA1A	HSPA1L	Coexpression	bioinformatics.ubc.ca/Gemma/mainMenu.html
HSPA1B	HSPA4	Other	18759004
HSPA4	BAG1	Regulation	9305631,10671488,11909948,14532969
HSPA4	BAG1	Physical Interaction	11231577,11101523,9679980,11527400,9305631,9873016, 16292491,14978028,15831476,14532969
HSPA4	BAG1	Other	16292491,11230127,17650072
HSPA4	HSF1	Up-Regulation	15081420,17273789
HSPA4	HSF1	Physical Interaction	9499401,17897941,17213196
HSPA4	HSF1	Other	12621024,17044073
HSPA4	HSF1	Predicted TFactor Regulation	
HSPA4	HSP90AA1	Physical Interaction	15850399,15632128,15795242,16314389,8603045
HSPA4	HSP90AA1	Other	15777846,12161444,16158055,8114727, 16682002,8776728,11751878
HSPA4	HSPA8	Coexpression	bioinformatics.ubc.ca/Gemma/mainMenu.html
HSPA4	HSPA8	Physical Interaction	11679576,16239242,15358145
HSPA4	HSPA8	Other	15596450,11222862,12874020
HSPA4	HSPA9	Coexpression	bioinformatics.ubc.ca/Gemma/mainMenu.html
HSPA5	HSPA4	Other	9409741
HSPA8	BAG1	Regulation	9305631,11707401,10671488,9321400,14532969
HSPA8	BAG1	Predicted Protein Interaction	10200254,10573421
HSPA8	BAG1	Physical Interaction	11101523,9305631,9873016,15986447, 14978028,9321400,14532969
HSPA8	HSF1	Physical Interaction	7639722
HSPA8	HSF1	Predicted TFactor Regulation	
HSPA8	HSP90AA1	Predicted Protein Interaction	10200254,10573421
HSPA8	HSP90AA1	Physical Interaction	9269769
HSPA8	HSP90AA1	Other	15358145,12220519
HSPA8	HSPA1A	Physical Interaction	11741305,9305631
HSPA8	HSPA9	Coexpression	bioinformatics.ubc.ca/Gemma/mainMenu.html
HSPA9	TP53	Down-Regulation	11900485
HSPA9	TP53	Physical Interaction	16176931,11900485
STUB1	HSPA1A	Physical Interaction	10330192
STUB1	HSPA4	Down-Regulation	10330192
STUB1	HSPA4	Physical Interaction	12574167,14962978,17545168,15845543,15761032,15254225
STUB1	HSPA4	Other	12150907,15845543
STUB1	HSPA8	Down-Regulation	10330192
STUB1	HSPA8	Predicted Protein Interaction	10200254,10573421
STUB1	HSPA8	Physical Interaction	15215316,10330192,15708501,15046863,15694383, 16111477,17324930,17963781,17157513,17545168, 16725394,16280320,15611333,18388150
STUB1	HSPA8	Other	14612456,16169850
TP53	HSPA1A	Physical Interaction	7811761
TP53	HSPA4	Up-Regulation	8940078,17278883
TP53	HSPA4	Physical Interaction	9235949,11507088,16909106
TP53	HSPA8	Physical Interaction	15911628,11707401,11297531

BAG1-BCL2-associated athanogene; STUB1-STIP1 homology and U-Box containing protein 1; TP53- Tumor protein p53

Figure 3: Interactions of HSP 70- genes predicted with GNC Pro Navigator.



From this interaction we selected Hsp5 because it is associated with Diabetes (REACT:15380) and Homeostasis (REACT:604) pathway described in CTD.[11] We made comparison of these results with Hspa 8 – a gene expressed in unstressed cells. As quantifying the exact metabolite using standards is resource intensive, we did *in silico* mining for metabolites specific to HSPA5 and HSPA8 genes as given in Polysearch database which displayed HMDB metabolite IDs as matches with Z score-normalized values (Table 2).

Though many study emphasize the anti–cell death mechanisms evidenced in HSP70 over expression, this work aimed at understanding expression-associated 'Gene Interaction for Cell survival' in manipulating the toxicity of different proteolytic enzymes in *Calotropis* sp through HSP by acting as molecular chaperons (intracellular housekeeping of proteins). To achieve this, besides studying HSP 70 gene interactions, the fine mapping of gene region to find out Probe ids (specific for HSP 70 genes involving reported gene associations) will be important for custom design of arrays or candidate marker for disease association/toxicity studies. Analysis of expression profiles currently available in different databases allows the monitoring of the extreme diversity encountered in different organism. Yet the use of microarrays that are suitable in *Calotropis* and/or Fructose fed rats must evaluate both the complex samples like *Calotropis* latex as well as that of Fructose, in their functional capacities/toxicities. Hence we used Genevestigator, wherein the array set specific to Wistar rats was available. In Genevestigator the intensity values of groups of probe IDs that cluster in relation to a given gene or set of genes in same category could be specifically evaluated as user defined parameters. Here we predicted the expression levels of Hspa5 in Genevestigator and the results are given in Figure 4 and 5. The Probe ID- 1370283_at showed 99.6% expression potential in 167 samples of Wistar rat Array whereas M14050_s_at showed 99.7% in 401 samples. Using multiple runs of Genevestigator, all possible array selection which is relevant to evaluate the combined dose fed rat was made for understanding different criteria (conditions, anatomy and stages of development) in altering HSP activity for handling *Calotropis* toxicity.(Data not shown) Probe IDs found associated with a given genes category are dynamically linked to facilitate tabular and graphical depiction of Entrez Gene information, Gene Ontology information, KEGG metabolic pathway diagrams and intermolecular interaction information which can be then used to develop a metagene (a set of genes) whose combined expression is linked to a specific cellular pathway.

Figure 4: Differential Expression of Hspa5 gene in Wistar Rat.

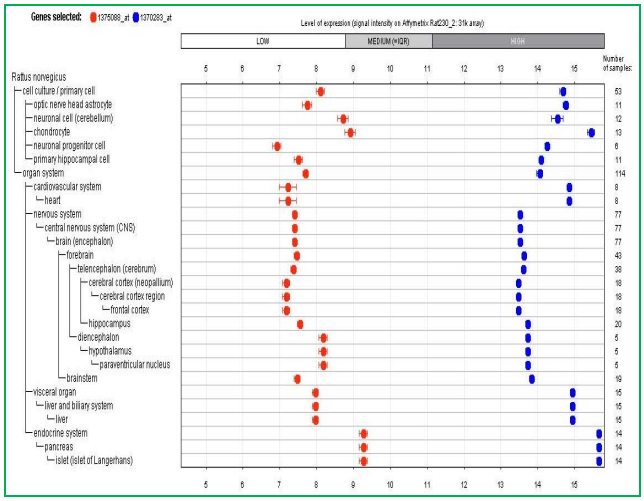
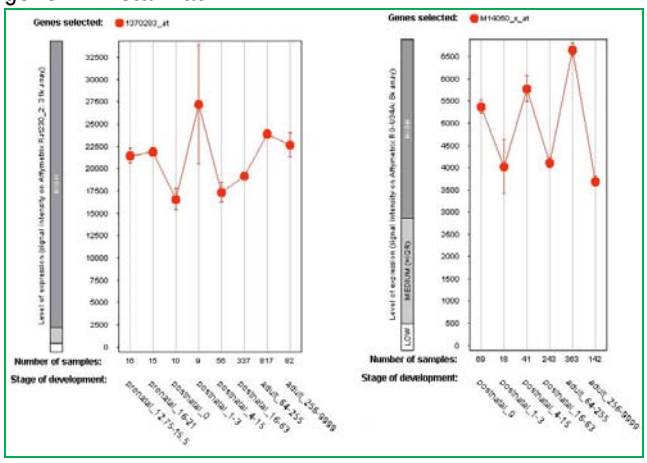


Figure 5: Array Specific Differential Expression of Hspa5 gene in Wistar Rat.



CONCLUSION

In conclusion, besides conserving resources, the relevant details required for this study were suitably harvested using different bioinformatics tools and databases containing references for data mining. The predictive approach shown here is to explain available gene function information and to provide robust prediction of

expression levels in new data.[25] We are undertaking further high throughput array experiments with defined gene regions and custom designed probes to elucidate the mechanism and efficacy of combined dose of *Calotropis* and Fructose -a strategy - that may provide promising evidence in near future in handling *Calotropis* latex toxicity and/or drug discovery.

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