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In Silico Analysis of Micro Propagated Endangered Species Costus Igneus

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Abstract: ***The present study was established to establish a protocol for in vitro micropropagation of Costus igneus and to investigate anti diabetic activity through in silco analysis. Direct Shoot regeneration occurred using the nodal and rhizome part of explants in MS medium supplemented with BAP (1 mg/l) + NAA (0.5 mg/l) show 77% shoots induction with 6.1cm in length. Among them the plant growth regulators (PGRs) BAP (1mg/l) and NAA (1mg/l) supplemented with MS medium had the maximum rooting about 78% and 11.5±0.04 no of roots. The acclimatization of rooted plants in ex vitro conditions was carried out with the plants bearing well-developed roots transferred to small pots containing soil mixtures (organic soil mixed with garden soil 1:1).***

They were maintained at about 70% relative humidity in the greenhouse with 75% shading. A survival rate 80 % was achieved after 6 weeks. The integration of computational and experimental strategies was great value in the identification and development of novel promising compounds.

Broadly used in modern drug design, molecular docking methods explore the ligand conformations adopted within the binding sites of macromolecular targets.

Phytocompounds present in the plant was done to find the appropeate ligand against INS and TNF α which were important genes associated with diabetes. From the docking results the best ligand was found to be strictinin with -12.17 binding energy when interacted with INS and -10.79 binding energy when interacted with TNF α.

Keywords: Micropropagation, Binding Energy, Strictinin, Ligands, Phytocompounds.

I. INTRODUCTION

Costus pictus D. Don (syn. Costus igneus Nak, Costus mexicanus or Costus congenitus), a perennial herbs belonging to Costaceae (Zingiberaceae) family, grows up to height of two to three feet. Popularly known as Insulin plant where as its botanical name is Costus pictus D. Don (Bakrudeen and Arun, 2009). The flowers appear on branches tip and orange in colour, ultimately develops into cone like fruits (Gilman, 2012). It originally belong South and Central America (Mexico) and was introduced to India (Beena and Joji, 2012) . In southern India, it usually grows as an ornamental plant and its leave are used as a dietary supplement in the treatment of diabetes mellitus (Vishalakshi etal , 2006). Leaves of Costus igneus is known to be effectively used for treating diabetes by the tribal people of Kolli Hills of Namakkal district, Tamil Nadu (Elavarasi and Saravanan, 2012). Alternative methods like Plant Tissue Culture will be helpful to achieve large rate of multiplication which leads to its conservation. Very limited study for the micropropagation has been done and multiplication rate was not very satisfactory. The present experiment was conducted to establish a simple and efficient protocol for micropropagation of Costus igneus and emphasized to achieve higher rate of multiplication. In this present study were identified as targets for diabetics, so these were subjected to extensive computational study to predict good quality model using modeling techniques and computer aided active site prediction. Docking allows virtually screening of compounds and predicts the strongest binders based on various scoring functions. It explores ways in which two molecules, such as drugs and phytochemicals fit together and dock to each other well. The molecules binding to a receptor, inhibit its function, and thus act as drug.

II. MATERIALS & METHODS

A. *Micropropagation*

1) *Selection of mother plant:* Plant material was collected from green house of techtona Biotech Resource Center, Bhubaneswar, Odisha. Explants were taken from actively growing, healthy and disease free shoots and nodes having meristematic tissue and washed under running water for removal of soil debris. Then disinfectant detergent teepol was added with continues stirring for 10-15 mins. Then explants were washed with distilled water and fungicide 0.1% (w/v).Bavistine was added and kept for 10mins with frequent stirring After 10 -15 min explants were washed with double distilled water for 2-3 times.

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2) *Initial media preparation & Inoculation of Explants:* The explants were inoculated on MS media added with different combinations and concentrations of growth regulators. The media for initial inoculation was prepared in different combination and concentration of MS with BAP (0, 0.5, 1,2mg/l) singly and MS with BAP (0.5, 1, 1.5mg/l) and NAA (0.5, 1mg/l) with maintain Ph 5.7-5.8. Nodal and rhizome took as explants and dipped in these medium for 4 weeks.
3) *Shoot proliferation:* The explants initially inoculated were allowed to grow up to 4weeks. Further for shoot multiplication, shoots from initial inoculation were transferred to MS basal medium which were supplemented with various concentrations BAP (1, 2mg/l) + NAA (0.5-1 mg/l) and BAP (1.2 mg/l) + IAA (0.2 mg/l).
4) *Root induction:* The elongated and well developed shoots were transferred to rooting media. The rooting media were prepared with MS supplemented with various concentrations of BAP (1mg/l) with IAA (0.1, 0.5, 1mg/l), BAP (1mg/l) with IBA (0.1, 0.5, 1mg/l) and BAP (1mg/l) with NAA(0.1, 0.5, 1mg/l).
5) *Plant acclimatization & Hardening:* All the cultures had been incubated in culture room at 25 ± 2°C, light depth (3000lux) with a photoperiod of 16 hours with 60-70% relative humidity. The cultures were monitored and the statistics have been recorded at each week interval. After 15-20 days of way of life, the sufficient rooted plantlets had been dipped in bavistin solution for approx 2-3 minutes and planted cautiously inside the poly luggage containing soil mixtures (organic soil blended with lawn soil 1:1). They were maintained at about 70% relative humidity in the greenhouse with 75% shading to supply newer leaves / roots. Plants are maintained underneath shade with controlled temperature and humidity to produce more modern leaves/roots. They are ready to be transferred in open nursery.
6) *In silico analysis:* These following tools and software's were used for the analysis of drug target identification with its inhibitory phytochemical selection and also analyze the binding affinity between them.
7) *Retrieval and Selection of target protein:* DisGeNET is a comprehensive discovery platform designed to address a variety of questions concerning the genetic underpinning of human diseases. To analyse the involvement of accrued target genes in unique metabolic pathways of blood, liver and lungs most cancers the pathway analysis was executed through KEGG (Kyoto Encyclopedia of Genes and Genomes) database (https://www.kegg.jp/), which is a resource for understanding high-level functions and utilities of the biological system and the pathway map represents the molecular interaction network diagram to explore the genomic relationship between the genes and the species.
8) *Selection of Template Protein:* UniProt (http://www.uniprot.org/) is a freely accessible database of protein sequence and functional information, many entries being derived from genome sequencing projects. It contains a large amount of information about the biological function of proteins derived from the research literature. The template proteins were collected from PDB (URL: https://www.rcsb.org/) database through BLASTP analysis. The selected template were used to generate the three dimensional model of target proteins.
9) *Model Generation:* Modeller (https://salilab.org/modeller/download_installation.html)is a computer program that models three-dimensional structures of proteins and their assemblies by satisfaction of spatial restraints. Modeller is most frequently used for homology or comparative protein structure modeling: The user provides an alignment of a sequence to be modeled with known related structures and Modeller will automatically calculate a model with all non-hydrogen atoms (these structures are often homologs, but certainly don't have to be, hence the term "comparative" modeling).Here, the three dimensional model of selected target protein was generated through modeler 9.21 by following the procedures.
10) *Preparation of ligand structure:* A ligand is a substance that forms a complex with a biomolecule to serve biological purpose. In protein-ligand binding, the ligand is usually a molecule which produces a signal by binding to a site on a target protein. The phytochemicals present in the target plant i.e *Costus igneus* were evaluated and four phytochemical were selected. The structure of above phytochemicals were searched and downloaded from PubChem (URL: https://pubchem.ncbi.nlm.nih.gov/) and save as SDF file and later converted to PDF file through Online SMILES Translator and Structure File Generator. Structure, bioactivity data as well as links to biological property information in PubMed and NCBI's Protein 3D Structure Resource.
11) *Docking Analysis:* Computational docking can be used to predict bound conformations and free energies of binding for small molecule ligands to macromolecular targets. Docking is widely used for the study of bio molecular interactions and mechanisms, and is applied to structure-based drug design. The methods are fast enough to allow virtual screening of ligand libraries containing tens of thousands of compounds. This protocol covers the docking and virtual screening methods provided by the AutoDock suite of programs. In this study, the structure based drug design and binding energy between small molecular ligands to macromolecule targets was done through Auto Dock programs. All the images were taken using PyMol (www.pymol.org) software after in computational approaches.

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III. RESULT

A. Explants Establishment And Shoot Initiation

It was observed that rhizomes were most suitable for in vitro propagation in *Costus igneus*. Out of different concentrations of BAP (0, 0.5, 1,2mg/l) singly and BAP (0.5, 1,1.5mg/l) with NAA (0.5, 1mg/l), the best result was seen in 1mg/l BAP+ 0.5mg/l NAA with 77% of shoot induction and 3.2 ± 0.04 average shoots per explant in rhizome followed by 1.5mg/l BAP + 0.5 mg/l NAA with 70% shoot induction and average 2.5±0.10 shoots per explants from stem node (table-1). Studies of Khaleghi et al. 2008, on Alstroemeria cv. "Fuego" showed that the greatest number of shoots was obtained from the medium supplemented with 1.5 mg /l BAP and 0.2 mg /l NAA. Results showed Largest number of shoot (3.00) and rhizome (4.00) obtained in MS medium containing 0.20 and 0.50 mg/l NAA Seyyedyousefi etal, 2013 was observed direct multiple shoots at BAP 2.0 mg/l + NAA 1.0 mg/l Ramar etal, (2014).

Table 1: Effect of PGRs on shoot Initiation:

Explants segment	Growth regulator BAP concentration (mg/l)	Growth regulator NAA concentration (mg/l)	Shoot induction %	Average no of shoots per explants
Stem Node	0	0	0	0
	0.5	0	20	1±0.12
	1	0	70	2.2±0.04
	2	0	65	2±0.03
	0.5	0.5	40	1±0.08
	1	0.5	70	2.5±0.10
	1.5	0.5	66	1.2±0.05
	0.5	1	35	1.1±0.11
	1	1	60	1.7±0.20
	1.5	1	60	1.2±0.12
Rhizome	0	0	0	0
	0.5	0	30	1±0.02
	1	0	72	2.3±0.09
	2	0	60	2±0.03
	0.5	0.5	0	0
	1	0.5	77	3.2±0.04
	1.5	0.5	70	2.5±0.10
	0.5	1	30	1±0.04
	1	1	55	1.5±0.13
	1.5	1	50	1.2±0.06

B. Shoot Multiplication And Elongation

The shoot length of *Costus ignius* was maximum in medium supplemented with 1 mg/l BAP+ 0.2mg/l IAA with 70% explants showing shoot proliferation , 4.2 ± 0.02 average shoots per explants and 6.1±0.04 cm average shoot length(Table-2). The optimal BAP concentration for shoot elongation was 1.0 mg/l. Experiments done by (Singh et al, 2014) showed BAP (2.5 mg/ l) and IAA (0.2 mg/l) induced highest rate (100 ± 2.66%) of regeneration with (23.2 ± 2.66) shoots per explants in *Psopho carpustetragonolobus* (L.) plant. Similarly the highest values of length and number of shoots produced were obtained in the media supplemented with 1.0 mg/l BAP + 0.5 mg/l IAA in the studies done by (Shamsiah et al, 2010). Similarly best suitable combinations of growth regulators recorded for Micropropagation by (Biradar et al, 2013), were 2.7 mg/l BAP, 0.2 mg/l IAA.

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Table- 2: Effect of various hormones on Shoot multiplication and elongation:

BAP (mg/l)	NAA(mg/l)	IAA(mg/l)	% of explants showing shoot proliferation	Average number of shoots per explant (mean ± SD)*	Average length of shoot (cm)*
0	0	0	20	1.1± 0.22	1.6±0.03
1	0.5	0	55	2.0 ± 0.01	3.5±0.04
2	1	0	65	3.5 ± 0.12	4.6±0.12
1	0	0.2	70	4.2 ± 0.02	6.1±0.04
2	0	0.2	60	2.2± 0.04	3.6±0.05

C. Rooting

The basal MS Media supplemented with 1mg/l BAP with IAA or IBA or NAA affected rooting in *Costeus ignius* shoots differently. Among the growth regulators, NAA had the largest effect on root formation with 78% of root explants and the numbers of roots were 11.5 ± 0.04 roots/explants (table- 3). NAA is a suitable for root induction that is consistent to **(Lin et al, 2000)** reports who suggested that NAA is an effective growth regulator for rooting. Similarly, **(Kristiansen et al, 1999)** reported that NAA promote root induction.

Table- 3: Effect of various hormones on rooting:

Hormones (mg/l)	Rooting (%)	Average no of roots per explant	Root length(cm) Mean±S.E
1.0 BAP+1.0 IAA	30.75	3.3±0.22	1.2±0.01
1.0 BAP+0.1 IBA	48.75	4.6±0.04	2.7±0.20
1.0 BAP+0.5 IBA	72.25	10.2±0.13	5.1±0.17
1.0 BAP+1.0 IBA	47.50	4.3± 0.08	2.60±0.29
1.0 BAP+0.1 NAA	35.25	3.5±0.10	1.5±0.25
1.0 BAP+0.5 NAA	78.50	11.5±0.04	5.5±0.04
1.0 BAP+1.0 NAA	50.75	4.5±0.12	2.5±0.15

IV. ACCLIMATIZATION & HARDENING

In vitro plantlets were transferred to soil rite for 15 to 17 days and subsequently transferred to polypots containing soil, sand and cow-dung manure at the ratio of 1:1:1 (v/v). All the regenerated plantlets were kept in the greenhouse with 65% relative humidity to produce newer leaves/roots. About 80 % of the plantlets established within 15 days of transfer. The plants grew well and attained 4–5 cm height within 1 months of transfer.

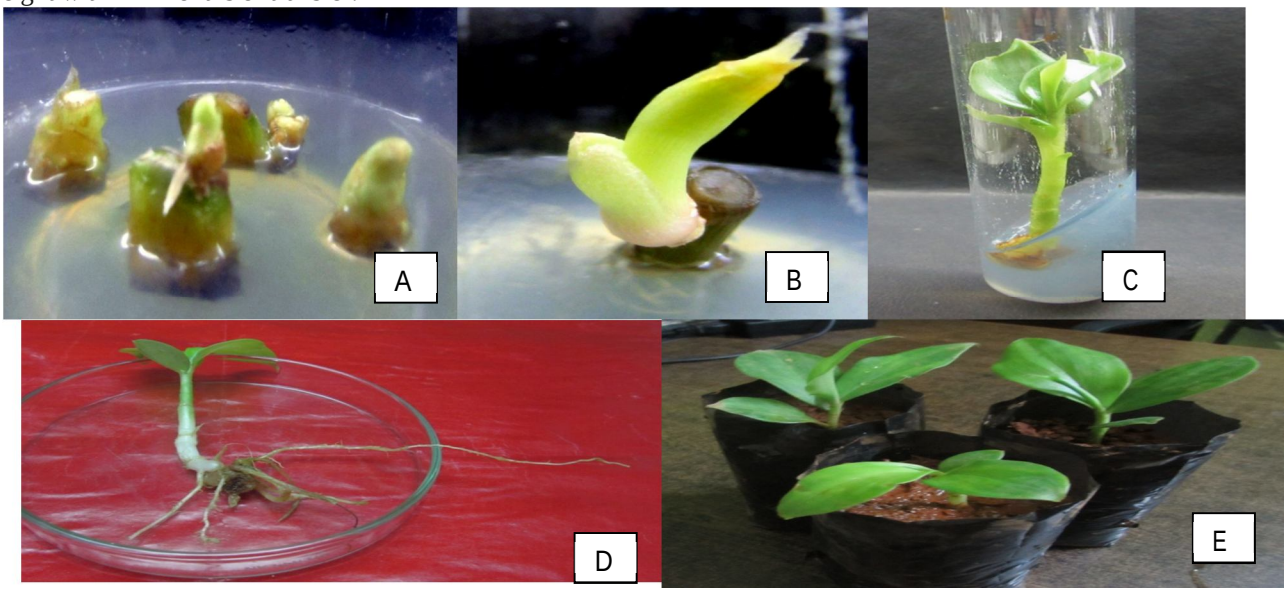


Fig 1: Showing (A) Initial Culture of Callus, (B) Shoot bud Initiation, (C) Multiplication & shoot elongation, (D) Root Initiation, (E) Hardening.

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V. COMPUTATIONAL ANALYSES

A. Collection of Candidate Genes

As it was discussed above DisGeNET, is a discovery platform containing one of the largest publicly available collections of genes and variants associated to human diseases. In this study, there were 2 types of Diabetes i.e, diabetes mellitus type 1and diabetes mellitus type 2 were collected top 8 numbers of putative target genes and association scores from the DisGeNET database (Table 4 & 5).

Table 4: Top 8 genes of diabetes mellitus type 1 (also known

Gene	Gene Name	Score
INS	Insulin	0.625
PTPN22	Protein tyrosine phosphatase non– receptor type 22	0.598
HNF1A	HNF1 homeobox A	0.442
FOXP3	forkhead box P3	0.418
HLA-DRB1	major histocompatibility complex, class II, DR beta1	0.365
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	0.331
TNF	tumor necrosis factor	0.309
HLA-DQA1	major histocompatibility complex, class II, DQ α 1	0.304

As insulin dependent)

Table 5: Top 8 genes of diabetes mellitus type 2 (also known as non-insulin dependent)

Genes	Gene Name	Score
GCK	Glucokinase	0.899
HNF1A	HNF1 homeobox A	0.812
HNF4A	hepatocyte nuclear factor 4 α	0.729
HNF1B	HNF1 homeobox B	0.684
AKT2	AKT serine/threonine kinase 2	0.681
ABCC8	ATP binding cassette subfamily C member 8	0.677
IRS1	insulin receptor substrate 1	0.67
NEUROD1	neuronal differentiation 1	0.645

Again, the pathway analysis was conducted to screen out the putative drug targets and their involvement in important and unique pathways of the different human diseases. In Diabetes mellitus type 1 (also known as type 1 diabetes), Diabetes mellitus type 2 (also known as type 2 diabetes) were collected 23 target genes, 26 number of genes and 35 numbers of targeted genes respectively(Fig-2), were analyzed from KEGG database. From total the most important 2 genes i.e. INS and TNF-α, which were involved in causing diabetes, were selected for further study.

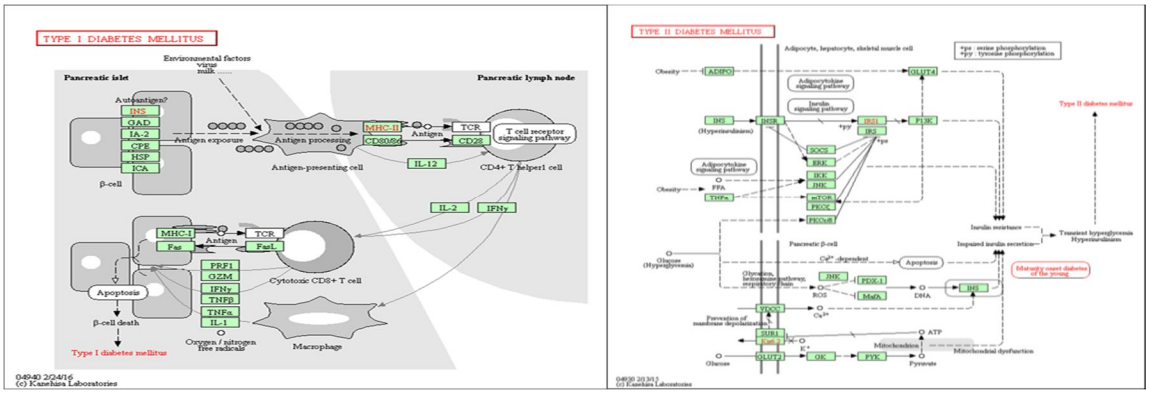


Fig 2**:** KEEG pathway of type I & type II diabetes mellitus

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These genes under gone deep analysis and searched the proteins that were encoded in those genes and their amino acid sequence length of those proteins were recorded from another online database Uniprot KB web server (https://www.uniprot.org/help/uniprotkb). Insulin (INS) and Tumor necrosis factor (TNF α) which were important and common genes found in almost all types of diabetes diseases (Table-6) , so from the above analyzed results there two genes were selected as candidate target genes for Diabetes.

Table-6: Information of genes collected from Uniport KB

Serial Number	Gene	Protein	Uniport ID	Sequence Length	Disease name
1.	INS	Insulin	P01308 (INS_HUMAN)	110	Diabetes
2.	TNF α	Tumor necrosis factor	P01375 (TNFA_HUMAN)	233	

B. Identification of Potent drug Target and Domain Prediction

Further, template structure identification for the three dimensional model generations the template selection process was done through BLASTP Program which was based on the high identity value and also the query coverage. The selected homologous proteins for target genes INS and TNF α were analyzed and the three dimensional structures of the selected template were retrieved from RCSB PDB i.e. for INS with PDBID 2KQP and human TNF-α in complex with jnj525, PDB: 5MU8 respectively**.**

Both these template proteins were undergone molecular modeling process for the generation of the three dimensional structure of the target protein INS and TNF- through the homology modelling software modeler, which predicts five numbers of three dimensional models for given target genes and the resulted best models for both the target proteins INS and TNF , obtained based on the dope score i.e. the resulted DOPE score for INS was (-7618.16504) and for TNF- was (-17351.24023) in comparison to other generated models.

The selected best models were again validated through energy minimization process; because Mod Refiner draws the initial starting models closer to their n ative state, in terms of hydrogen bonds, backbone topology and side-chain positioning. It also generates significant improvement in physical quality of local structures. So here the energy minimization was done through Mod refiner and the validated or refined structures of INS and TNF- were going for further analysis.

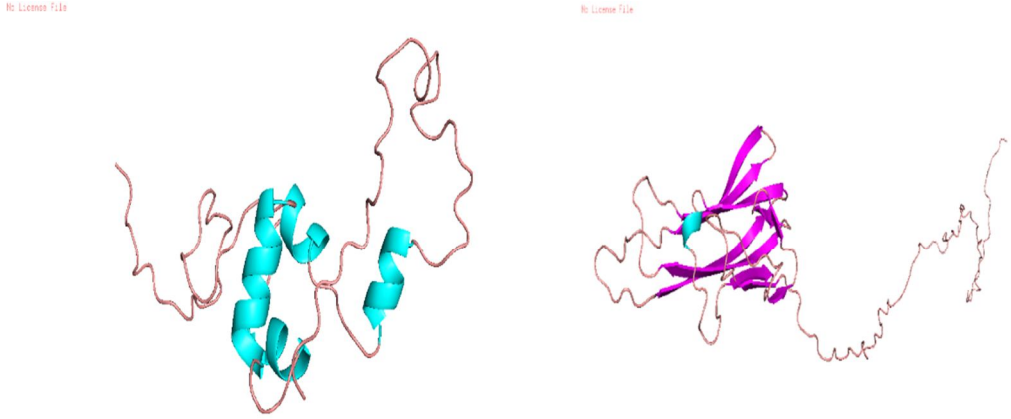


Fig.-3 Best model structure of INS and TNF proteins respectively according to their dope score.

C. Selection of Natural Compounds

As per literature survey, that are present in the plant *C. igneus* in a good quantity that have potential anti carcinogenic, anti-cancerous or antitumor properties were collected six natural compounds such as Epicatechin, Strictinin,Quercetin and Diosgeninhave been identified with medicinal properties and reported useful against different infectious diseases (Table-7), and hence downloaded from PubChem database of NCBI web server

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Table-7: Collected information about selected four natural compounds against *C. igneus*

Serial Number	Chemical Name	Molecular Formula	Pubchem id	Activities
1.	Epicatechin	$C_{15}H_{14}C_6$	72276	Epicatechin is a strong antioxidant, has insulin mimic action and improves heart health.
2.	Strictinin	$C_{27}H_{22}O_{18}$	73330	Strictinin is a bioactive chemical of the ellagitannin family of hydrolyzabletannins.
3.	Quercetin	$C_{21}H_{20}O_{11}$	5280459	Quercetin supplements have been promoted for the treatment of cancer and various other diseases.
4.	Diosgenin	$C_{27}H_{42}O_3$	99474	Diosgenin, a phytosteroidsapogenin, is the product of hydrolysis by acids, strong bases, or enzymes of saponins.

VI. MOLECULAR DOCKING ANALYSIS

The results of molecular docking were intended for energetically favorable binding poses for each four natural compounds proposed drug target of *C. igneus*. The best binding interaction was seen by (Insulin) INS with compound strictinin. The binding energy of this docking result -12.17, which was the best binding energy, based on docking algorithms, resulted from all of the docking processes. Again, in docking results Epicatechin showed the best docking energy (-3.48 with INS and -6.44 with TNF α) found with lower binding energy and inhibition constants 2,82mM (INS), and 18.9uM (TNF α)followed by Strictinin (-12.17 with INS and -10.79 with TNFα)found with lower binding energy and inhibition constants 1.2nM (INS) and 12.41nM, Quercetin (-5.19 with INS and -5.41 with TNF)found 158.17mM and 107.53uM, Diasgenin (-8.99 with INS and –8.17 with TNF) found with lower binding energy and inhibition constants 257.9 nM and 1.02 uM respectively (Table-8).So this compound strictinin could be used as an inhibitor to mutated (Insulin) INS gene as it has good binding affinity towards this gene.

Table-8: Information obtained from docking calculation between selected natural compounds and drug target (INS and TNF α)

Phytocompound names	Gene name	Binding energy	KI	Intermolecular Energy	Cluster RMS	Ref RMS
Epicatechin	INS	-3.48	2.82mM	-5.27	0.0	16.78
	TNF α	-6.44	18.9uM	-8.23	0.0	31.95
Strictinin	INS	-12.17	1.2nM	-15.75	0.0	10.11
	TNF α	-10.79	12.41nM	-14.37	0.0	29.98
Quercetin	INS	-5.19	158.17mM	-6.98	0.0	15.56
	TNF α	-5.41	107.53uM	-7.2	0.0	35.35
Diosgenin	INS	-8.99	257.9Nm	-9.29	0.0	27.85
	TNF α	-8.17	1.02uM	-8.47	0.0	32.65

So, these results showing the good inhibitory effect against Diabetes and revels strictinin is the best inhibitor against INS and TNF α were having the better binding affinity i.e. -12.17 and -10.79 respectively to cure diabetes.

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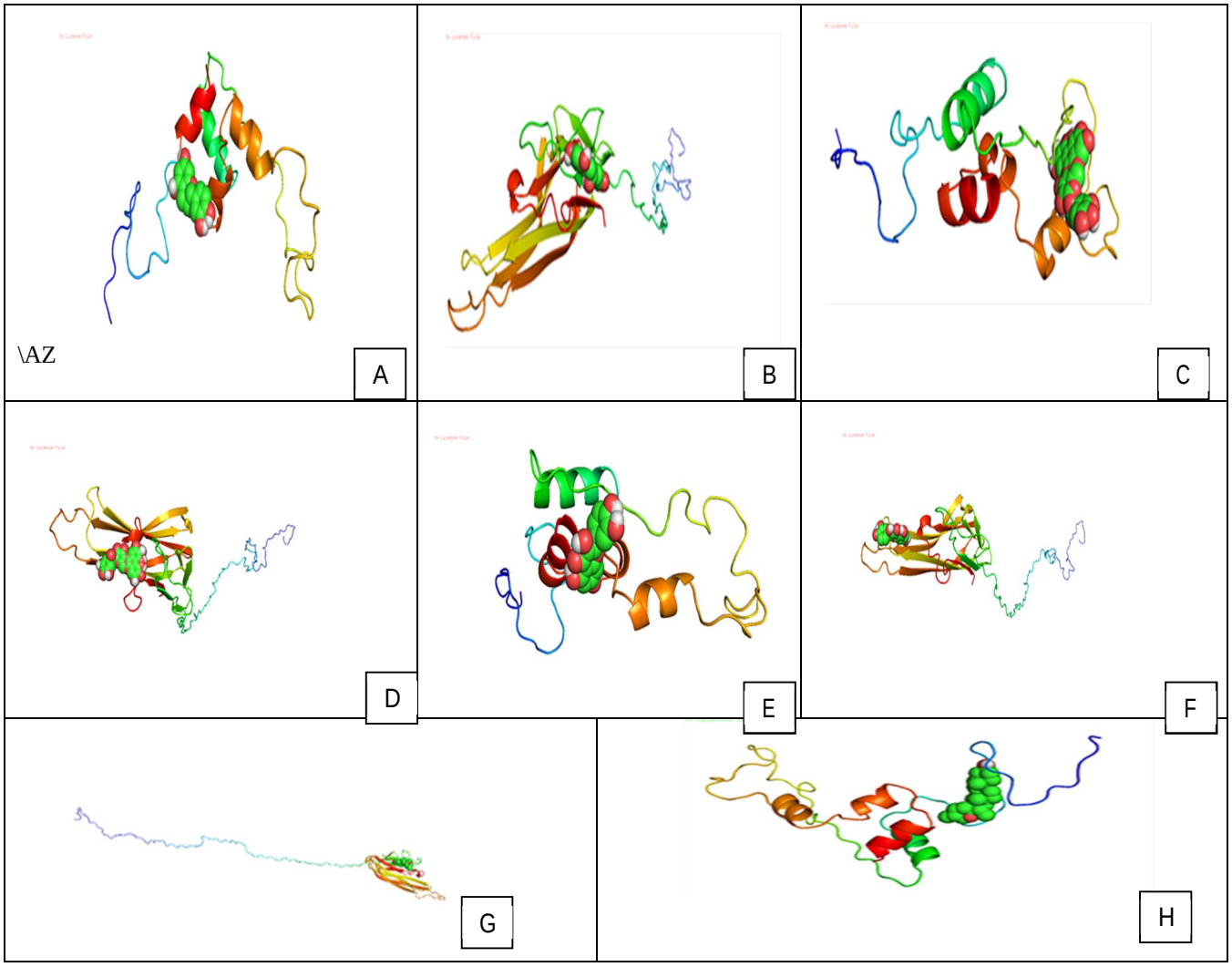


Fig.-4: Interaction profile of six natural compounds with proposed drug target: INS – Epicatechin (A), TNF α Epicatechin (B), INS-Strictinin (C), TNF α- Strictinin(D), INS- Quercetin (E), TNF α –Quercetin (F), INS-Diosgenin (G), Diosgenin deciphered, respectively.

VII. DISCUSION

From these experiments it should observed that the plant *Costus igneus* is an important plant for cure of diabetes and it is an endangered plant. So one simple micropropagation protocol was standardized and performs drug design to cure diabetes using tool molecular modeling and docking. From (table 1) it was observed that, the greatest number of shoots 70% and 77% was obtained from the medium supplemented with 1 mg /l BAP and 0.5 mg /l NAA in both rhizomes and stem node with 3.2±0.04 and 2.5±0.10 average shoot per explants respectively. From (table 2) , 70% of explants from both rhizome and stem node with **4.2 ± 0.02** average shoots per explants and **6.1**±0.04 cm average shoot length in the Ms media supplemented with BAP (1mg/l) and IAA (0.2mg/l). From (table 3) it was resulted that NAA was essential for root induction. All the regenerated plantlets were kept in the greenhouse with 65% relative humidity to produce newer leaves/roots. About 80 % of the plantlets established within 15 days of transfer. Pharmaceutical research has successfully incorporated a wealth of molecular modeling methods, within a variety of drug discovery programs, to study complex biological and chemical systems. The integration of computational and experimental strategies has been of great value in the identification and development of novel promising compounds. Broadly used in modern drug design, molecular docking methods explore the ligand conformations adopted within the binding sites of macromolecular targets. In the 2nd part of study the literature survey of the phytocompounds present in the plant was done to find the appropeate ligand against INS and TNF α which are important genes associated with diabetes. From the docking results the

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best ligand was found to be strictinin with -12.17 binding energy when interacted with INS and -10.79 binding energy when interacted with TNF α (Table-8).

VIII. CONCLUSION

Direct Shoot regeneration using the nodal and rhizome explants was observed in MS with BAP (1 mg/l) + NAA (0.5 mg/l) with 77% shoot induction. The maximum shoot length of *Costus igneus* was seen in medium supplemented with 1 mg/l BAP+ 0.2mg/l IAA with 70% explants showing shoot proliferation. Among the various growth regulators tested for rooting of the plant, MS with BAP (1mg/l) + NAA (1mg/l) was found to be best with 78% rooting. For acclimatization well grown plantlets were transferred to pots containing mixture of sand, soil and vermin compost (1:3:2) and showed 98% survival rate. In silico analysis, conclude that strictinin has the best binding affinity with diabetes protein which can be act as a drug for diabetes. More research and clinical trials are needed to approve the phytochemicals as anti-diabetics drugs.

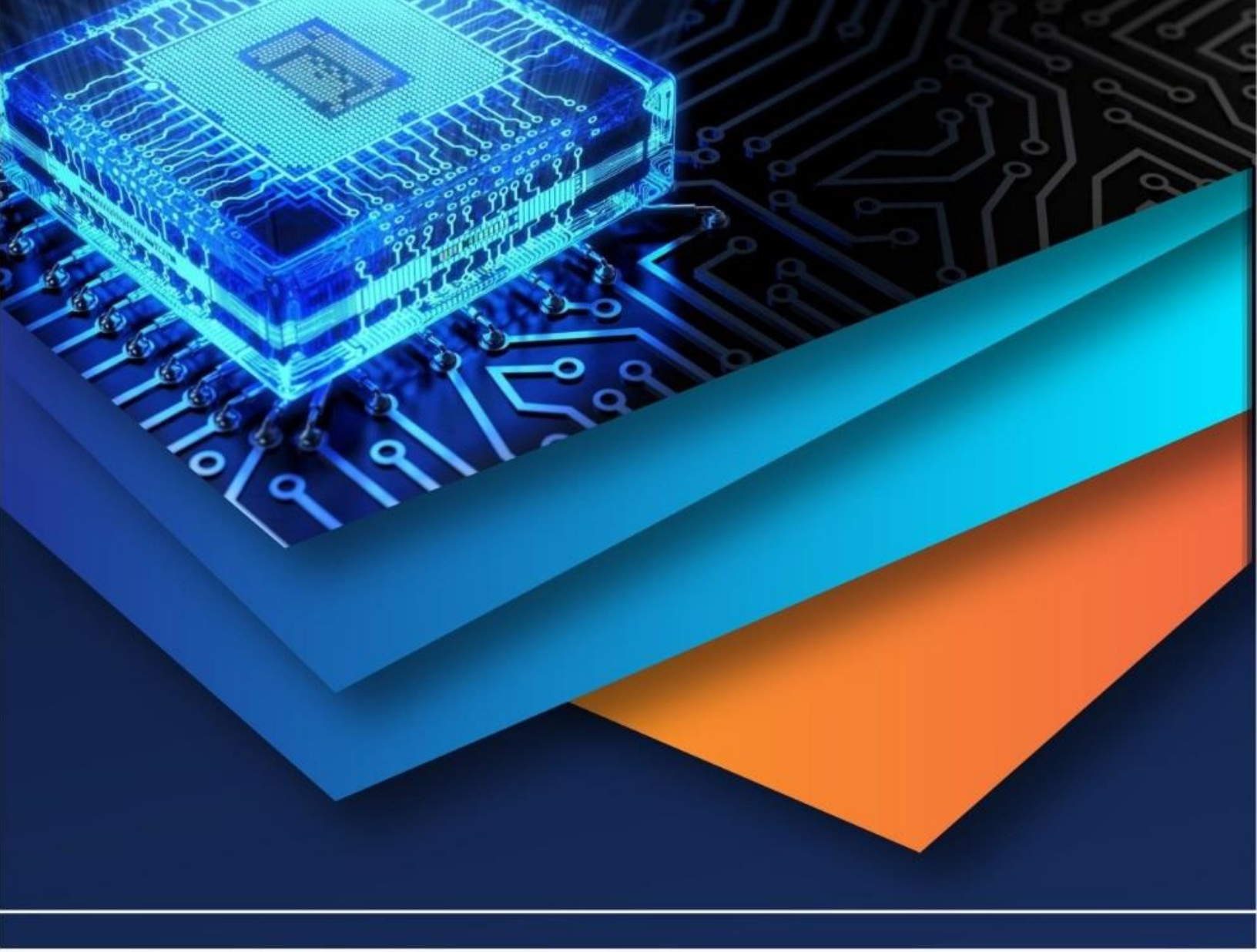
IX. ACKNOWLEDGEMENT

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