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Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. Regulatory Peptides, 139: 109-114.

Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4$^{th}$ edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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# In-silico analysis and homology modelling of antioxidant proteins present in *Pisum sativum*

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**Abstract**

Garden Peas are edible seeds that have an essential nutrient required for human diet. Peas contain phytochemicals that shows antioxidant activities. A computational approach was adopted to analyse the characteristic properties and structure of the Peas antioxidant proteins. Superoxidase dismutase (SOD), catalase, phospholipid hydroperoxide glutathione peroxidase-like protein (PHGPX) and ascorbate peroxidase (APX) are the antioxidant proteins which have been used in this study. Physiochemical properties such as isoelectric point, molecular weight, aliphatic index etc. were analysed by using the Expasy's Prot Param server. Secondary structure of these proteins was analysed to study the functional characteristic of the protein. The crystal structure of the ascorbate peroxidase was available, but the crystal structures of other proteins were not available in any of the protein structure database. Hence the three-dimensional structures of these proteins were generated using Swiss Modeller and Geno3D followed by validation through SAVES server. This analysis will provide additional help for the analysis of crystal structure for further experimentation.

## Introduction

*Pisum sativum* (Garden Peas) is the legume crop grown in different regions of the world. Amongst the world's pulses, it ranks second to dry beans in production and consumption (1). Besides from the rich in carbohydrates, vitamins, and minerals, proteins and fibre content, it has some medicinal value as well. The extract obtained from the seeds of the garden pea has been shown to have antibacterial (2), anti-inflammatory (3) and antioxidant properties in biological systems (4). Several successful studies on the antioxidant proteins have been conducted but there is still no detailed information about the sequence of the antioxidant proteins, structures and their mechanism of action (5).

Various biotic and abiotic stresses lead to the generation of reactive oxygen species (ROS) in plant cells as a first response towards the stress (6). Superoxide radicals (O-2), hydroperoxides (ROOH) and hydroxyl radical (OH-), H2O2 etc. are some reactive oxygen species that are formed as a by-products of aerobic energy metabolism. These reactive oxygen species causes oxidative damage like oxidation of proteins, enzyme inactivation, gene expression alteration that ultimately leads to cellular damage (7,8). Antioxidants are the small substances that delays, prevents or eradicates the oxidative damage to a target molecule (9). Superoxide dismutase (SOD), Catalase, phospholipid hydroperoxide glutathione peroxidase-like protein (PHGPx) and ascorbate peroxidase (APX) are the major oxygen radical detoxifying enzymes (10). In this study we have analysed the antioxidant proteins of Pisum sativum through in silico approaches. Only the three-dimensional structure of APX is available in protein databank (PDB), while there is no information about the other proteins viz., SOD, PHGPx and catalase.

## Material and Methods

Sequences of Pisum sativum antioxidant proteins were retrieved from UniProtKB/Swiss-Prot

which is freely accessible public database that is manually annotated. It can be accessed directly from the URL http://www.uniprot.org/. The proteins used in this study are indexed in Table 1. Protein sequences were retrieved in FASTA format which was further used for the study of the antioxidant proteins.

## Physicochemical characterization

Computational analysis for the study of the physico-chemical parameters of the antioxidant proteins were performed using the ExPASy World Wide Web server. It have several online tools that predicts certain properties of proteins like isoelectric point (pI), molecular weight (M. wt), peptide mass, amino acid composition, extinction coefficient(EC) (11), atomic composition, instability index (II), grand average hydropathy (GRAVY) (12) total number of positive and negative residues (+R & -R), etc. FASTA format of the protein sequence were used for the physico-chemical analysis using ProtParam tool.

## Functional characterization

SOSUI server was used to classify and predicts the secondary structure of a transmembrane protein. SOSUI searches for a (alpha) helix and predicts the transmembrane region of a protein. SOSUIsignal was used to identify the transmembrane protein, and the predicted transmembrane region of the protein is represented in Table

**Table 1:** Protein sequences used in the study.

| Antioxidant Proteins | Unique Accession No. | Length | Gene | Function | Description |
|---|---|---|---|---|---|
| SOD | P11964 | 202 | SODCP | It destroys the radicals naturally produced inside the cell that are toxic to biological systems. | Superoxide dismutase [Cu-Zn], chloroplastic |
| | Q02610 | 152 | SODCC | | Superoxide dismutase [Cu-Zn] |
| | P27084 | 233 | SODA | | Superoxide dismutase [Mn], mitochondrial |
| | Q5DWE8 | 152 | SOD | | Superoxide dismutase [Cu-Zn] |
| | Q7XHK3 | 243 | sodB | | Superoxide dismutase |
| | Q6TA12 | 161 | N/A | | Superoxide dismutase |
| | Q9SC38 | 195 | ger2b | | Germin-like protein |
| | Q9SC39 | 195 | ger2a | | Germin-like protein |
| Catalase | P25890 | 494 | *N/A* | It is present in all aerobic organisms and protects the cell from toxic effects of hydrogen peroxide. | Catalase |
| | C0STY9 | 494 | PCAT1 | | Catalase |
| PHGPx | O24296 | 236 | *N/A* | Prevents the cells as well as enzymes from oxidative damage. | Phospholipid hydroperoxide glutathione peroxidase, chloroplastic |
| APX | P48534 | 250 | APX1 | Removes the hydrogen peroxide. | L-ascorbate peroxidase, cytosolic |
| | C7EXK9 | 207 | stAPX | It is Heme binding protein which shows peroxidase activity | Chloroplast stromal ascorbate peroxidase 12 |

**Table2:** Parameter of protein computed using ProtParam Tool.

| Antioxidant Proteins | Accession No. | No. of Amino Acids | M. wt | pI | -R | +R | EC | II | AI | GRAVY |
|---|---|---|---|---|---|---|---|---|---|---|
| SOD | P11964 | 202 | 20626.2 | 5.94 | 17 | 12 | 1615 | 20.19 | 94.06 | 0.031 |
| | Q02610 | 152 | 15322.8 | 5.59 | 15 | 8 | 125 | 11.36 | 78.88 | -0.247 |
| | P27084 | 233 | 25822.4 | 7.16 | 24 | 24 | 46410 | 29.91 | 96.35 | -0.269 |
| | Q5DWE8 | 152 | 15322.8 | 5.59 | 15 | 8 | 125 | 11.36 | 78.88 | -0.247 |
| | Q7XHK3 | 243 | 27163.6 | 6.50 | 27 | 25 | 47440 | 33.13 | 76.34 | -0.411 |
| | Q6TA12 | 161 | 18298.6 | 5.88 | 21 | 16 | 45950 | 21.82 | 84.22 | -0.434 |
| | Q9SC38 | 195 | 21260.3 | 6.06 | 18 | 16 | 11585 | 15.59 | 100.41 | 0.082 |
| | Q9SC39 | 195 | 21244.3 | 6.06 | 18 | 16 | 11585 | 17.78 | 98.41 | 0.045 |
| CATALASE | P25890 | 494 | 57344.5 | 6.72 | 59 | 55 | 87570 | 41.55 | 69.66 | -0.589 |
| | C0STY9 | 494 | 57176.3 | 6.73 | 59 | 55 | 80705 | 40.82 | 70.24 | -0.575 |
| PHGPx | O24296 | 236 | 26400.1 | 9.39 | 21 | 30 | 13075 | 34.13 | 66.91 | -0.283 |
| APX | P48534 | 250 | 27192.7 | 5.52 | 36 | 28 | 21430 | 36.55 | 80.48 | -0.332 |
| | C7EXK9 | 207 | 22764.4 | 5.48 | 32 | 27 | 39420 | 39.35 | 64.20 | -0.752 |

5. After the prediction of transmembrane region, it was visualisedand analysed by helical wheel plot generated via Pepwheel 32 programme, a part of EMBOSS 6.5.7 suite (Figure.1).

For the determination of functional linkages, disulphide bonds are important (13). Tool DiANNA 1.1 (http://clavius.bc.edu/~clotelab/DiANNA/) web server was used to identify the disulphide bond in a protein sequence data. DiANNA (DiAmino acid Neural Network Application) is a webserver for prediction of cysteine classification and disulphide connectivity (14). Predicted disulphide bonds for all the antioxidant proteins are mentioned in Table 5. Prosite (http://www.expasy.org/prosite/) is a database that consists of documentation of protein domains and families. It also annotates the associated patterns and profiles of the protein (15). The output of the Prosite is represented in Table 6.

**Figure1:** Helical Wheel presentation of predicted transmembrane region of Q02610 and Q5DWE8. Hydrophobic residues (LVM) are represented as a blue square, violet letter (AGPY) and (HKR) in black octagons.

**Prediction of Secondary Structure of Protein**

The secondary structure of a protein was predicted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) that uses self-optimized prediction method (SOPM) and neural networks method (16). The secondary structural element was predicted and the results were displayed in Table 7.

**Modelling and Evolution of Protein Three-Dimensional Structure**

Three dimensional structure of Ascorbate peroxidase (APX) was available in PDB databank (www.rcsb.org), but the structure of Catalase, Superoxide dismutase (SOD), Phospholipid hydroperoxide glutathione peroxidase (PHGPx) were not available so we have modelled the structure of these antioxidant proteins by using two homology modelling program SwissModel (17), and Geno3D (18). Swiss Structure & Model Assessment Tools, a Swiss Model workspace was used to assess the modelled structure. Stereochemistry check was performed through the Procheck program (19). Rasmol was used to visualise the modelled structures (20).

**Result**

Detailed information about the antioxidant proteins of *Pisum sativum* were discussed in Table1.These proteins were retrieved from UniProtKB/Swiss-Prot, a freely accessible public database. FASTA format of the protein sequences

**Table 3**: Amino acid composition of antioxidant proteins of *Pisum sativum*.

| | Antioxidant Proteins | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Amino Acids** | **P11964** | **Q02610** | **P27084** | **Q5DWE8** | **Q7XHK3** | **Q6TA12** | **Q9SC38** | **Q9SC39** | **P25890** | **C0STY9** | **O24296** | **P48534** | **C7EXK9** |
| Ala (A) | 8.4% | 6.6% | 10.3% | 6.6% | 8.6% | 8.7% | 6.7% | 6.2% | 5.9% | 5.9% | 5.5% | 10.4% | 9.7% |
| Arg (R) | 2.5% | 2.0% | 2.6% | 2.0% | 2.5% | 1.9% | 1.5% | 1.5% | 7.1% | 7.1% | 2.1% | 3.6% | 4.8% |
| Asn (N) | 5.0% | 7.9% | 5.2% | 7.9% | 7.0% | 7.5% | 7.7% | 7.7% | 5.9% | 5.7% | 5.5% | 1.6% | 2.9% |
| Asp (D) | 4.0% | 5.9% | 4.7% | 5.9% | 4.9% | 6.2% | 7.2% | 7.2% | 6.3% | 6.3% | 4.7% | 6.8% | 7.7% |
| Cys (C) | 1.0% | 1.3% | 0.4% | 1.3% | 0.4% | 0.0% | 1.0% | 1.0% | 1.0% | 1.2% | 1.3% | 0.4% | 0.5% |
| Gln (Q) | 2.5% | 1.3% | 4.3% | 1.3% | 2.1% | 2.5% | 4.6% | 4.6% | 3.4% | 3.4% | 4.2% | 2.4% | 2.9% |
| Glu (E) | 4.5% | 3.9% | 5.6% | 3.9% | 6.2% | 6.8% | 2.1% | 2.1% | 5.7% | 5.7% | 4.2% | 7.6% | 7.7% |
| Gly (G) | 11.9% | 17.8% | 6.9% | 17.8% | 6.6% | 7.5% | 8.2% | 8.7% | 5.1% | 5.1% | 4.7% | 9.6% | 11.1% |
| His (H) | 4.5% | 5.9% | 4.3% | 5.9% | 3.7% | 5.0% | 2.1% | 2.1% | 5.5% | 5.7% | 0.4% | 3.2% | 1.9% |
| Ile (I) | 3.0% | 6.6% | 6.4% | 6.6% | 3.7% | 3.7% | 6.7% | 7.2% | 4.9% | 5.1% | 4.7% | 4.4% | 2.4% |
| Leu (L) | 10.9% | 6.6% | 11.2% | 6.6% | 10.3% | 10.6% | 9.7% | 9.2% | 6.7% | 6.5% | 6.4% | 10.0% | 8.7% |
| Lys (K) | 3.5% | 3.3% | 7.7% | 3.3% | 7.8% | 8.1% | 6.7% | 6.7% | 4.0% | 4.0% | 10.6% | 7.6% | 8.2% |
| Met (M) | 0.5% | 0.7% | 0.9% | 0.7% | 1.2% | 0.6% | 0.0% | 0.0% | 2.0% | 1.8% | 1.3% | 0.8% | 1.0% |
| Phe (F) | 2.5% | 2.6% | 1.7% | 2.6% | 6.2% | 5.0% | 7.7% | 7.7% | 6.3% | 6.3% | 11.0% | 5.6% | 3.4% |
| Pro (P) | 5.9% | 4.6% | 3.9% | 4.6% | 6.2% | 4.3% | 6.2% | 6.7% | 7.1% | 7.1% | 6.4% | 7.2% | 7.7% |
| Ser (S) | 8.4% | 5.3% | 6.0% | 5.3% | 8.2% | 4.3% | 4.1% | 4.1% | 6.1% | 6.3% | 11.4% | 5.6% | 4.8% |
| Thr (T) | 9.9% | 10.5% | 5.6% | 10.5% | 4.5% | 3.1% | 5.1% | 5.1% | 4.9% | 4.9% | 6.8% | 4.8% | 4.3% |
| Trp (W) | 0.0% | 0.0% | 2.6% | 0.0% | 2.9% | 4.3% | 0.5% | 0.5% | 2.2% | 2.0% | 0.4% | 0.8% | 2.4% |
| Tyr (Y) | 0.5% | 0.0% | 3.9% | 0.0% | 2.5% | 3.1% | 2.1% | 2.1% | 3.6% | 3.4% | 2.1% | 2.8% | 3.9% |
| Val (V) | 10.9% | 7.2% | 6.0% | 7.2% | 4.5% | 6.8% | 10.3% | 9.7% | 6.5% | 6.7% | 6.4% | 4.8% | 3.9% |
| Pyl (O) | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% |
| Sec (U) | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% |

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**Table 4:** Prediction of transmembrane protein and soluble protein.

| Antioxidant Proteins | Accession No. | N terminal | transmembrane region | C terminal | type | length | Description |
|---|---|---|---|---|---|---|---|
| SOD | Q02610 | 1 | MVKAVAVLSN | 10 | Signal Peptide | 10 | This amino acid sequence has signal peptide |
| | Q5DWE8 | 1 | MVKAVAVLSN | 10 | Signal Peptide | 10 | |
| | P11964 | - | - | - | - | - | This amino acid sequence has no signal peptide. (SOLUBLE PROTEIN) |
| | P27084 | - | - | - | - | - | |
| | Q7XHK3 | - | - | - | - | - | |
| | Q6TA12 | - | - | - | - | - | |
| | Q9SC38 | - | - | - | - | - | |
| | Q9SC39 | - | - | - | - | - | |
| CATALASE | P25890 | - | - | - | - | - | |
| | C0STY9 | - | - | - | - | - | |
| PHGPx | O24296 | - | - | - | - | - | |
| APX | P48534 | - | - | - | - | - | |
| | C7EXK9 | - | - | - | - | - | |

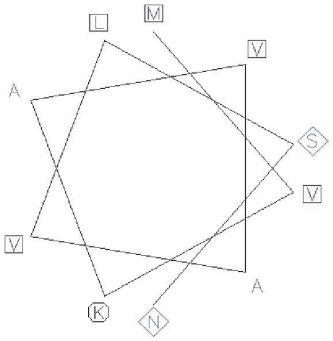


**Figure1:** Helical Wheel presentation of predicted transmembrane region of Q02610 and Q5DWE8. Hydrophobic residues (LVM) are represented as a blue square, violet letter (AGPY) and (HKR) in black octagons.

**Table 5:** Disulphide bond connectivity prediction in protein sequences.

| Antioxidant Proteins | Accession No. | Cysteine sequence position | Distance | Predicted Bond | Predicted connectivity |
|---|---|---|---|---|---|
| SOD | P11964 | Cys105-Cys194 | 89 | DTTNGCISTGP-GGRLACGVVGL | 1-2 |
| | Q02610 | Cys56 - Cys145 | 89 | DTTNGCISTGP - GGRVACGIIGL | 1-2 |
| | P27084 | - | - | No disulphide bonds are possible for this sequence | - |
| | Q5DWE8 | Cys56 - Cys145 | 89 | DTTNGCISTGP - GGRVACGIIGL | 1-2 |
| | Q7XHK3 | - | - | No disulphide bonds are possible for this sequence | - |
| | Q6TA12 | - | - | No disulphide bonds are possible for this sequence | - |
| | Q9SC38 | Cys4 - Cys21 | 17 | XXQDFCVAIKD - VNGKFCKDPAL | 1-2 |
| | Q9SC39 | Cys4 – Cys21 | 17 | XXQDFCVAIKD - VNGKFCKDPAL | 1-2 |
| CATALASE | P25890 | Cys230 - Cys325 | 95 | HWKPTCGVKCL – EQLAFCPAIML | 2-4 |
| | | Cys234 - Cys420 | 186 | TCGVKCLLEEE - ARREKCNIPKQ | 3-5 |
| | C0STY9 | Cys86 - Cys370 | 284 | ISHLTCADFLR -VNAPKCSHHNN | 1-5 |
| | | Cys230 - Cys325 | 95 | HWKPTCGVKCL - EQLAFCPAIIV | 2-4 |
| | | Cys234 - Cys420 | 186 | TCGVKCLLEEE - ARREKCNIPKQ | 3-6 |
| PHGPx | O24296 | Cys111 - Cys159 | 48 | NVASRCGLTSS - IKQFACTKFKA | 1-3 |
| APX | P48534 | - | - | No disulphide bonds are possible for this sequence | - |
| | C7EXK9 | - | - | | - |

**Table 6:** Functional characterisation of Antioxidant Protein using Prosite database.

| Antioxidant Proteins | Accession No. | Motif | Profile | Position in the protein |
|---|---|---|---|---|
| SOD | P11964 | - | SOD_CU_ZN_1<br>SOD_CU_ZN_2 | 92 – 102<br>186 - 197 |
| | Q02610 | - | | 43 - 53<br>137 - 148 |
| | P27084 | - | SOD_MN | 194 - 201 |
| | Q5DWE8 | - | SOD_CU_ZN_1<br>SOD_CU_ZN_2 | 43 – 53<br>137 - 148 |
| | Q7XHK3 | - | - | - |
| | Q6TA12 | - | SOD_MN | 153 – 160 |
| | Q9SC38 | - | GERMIN | 76 - 89 |
| | Q9SC39 | - | GERMIN | 76 - 89 |
| CATALASE | P25890 | CATALASE_3 | CATALASE_2<br>CATALASE_1 | 14 – 494<br>54 – 70<br>344 - 352 |
| | C0STY9 | CATALASE_3 | CATALASE_2<br>CATALASE_1 | 54-70<br>344 - 352 |
| PHGPx | O24296 | GLUTATHIONE_PEROXID_3 | GLUTATHIONE_PEROXID_1<br>GLUTATHIONE_PEROXID_2 | 99 – 114<br>136 - 143 |
| APX | P48534 | PEROXIDASE_4 | PEROXIDASE_2<br>PEROXIDASE_1 | 74 – 250<br>33 – 44<br>155 - 165 |
| | C7EXK9 | PEROXIDASE_4 | PEROXIDASE_1 | 55 – 207<br>123 - 133 |

**Table 7:** Secondary structure prediction using SOPMA tool.

| Accession No. | Alpha helix | $3_{10}$ helix | Pi helix | Beta bridge | Extended strand | Beta turn | Bend region | Random coil | Ambiguous states | Other states |
|---|---|---|---|---|---|---|---|---|---|---|
| **P11964** | 18.81% | 0.00% | 0.00% | 0.00% | 33.17% | 9.90% | 0.00% | 38.12% | 0.00% | 0.00% |
| **Q02610** | 5.26% | 0.00% | 0.00% | 0.00% | 33.55% | 8.55% | 0.00% | 52.63% | 0.00% | 0.00% |
| **P27084** | 38.20% | 0.00% | 0.00% | 0.00% | 20.60% | 12.45% | 0.00% | 28.76% | 0.00% | 0.00% |
| **Q5DWE8** | 5.26% | 0.00% | 0.00% | 0.00% | 33.55% | 8.55% | 0.00% | 52.63% | 0.00% | 0.00% |
| **Q7XHK3** | 28.81% | 0.00% | 0.00% | 0.00% | 20.99% | 5.35% | 0.00% | 44.86% | 0.00% | 0.00% |
| **Q6TA12** | 42.24% | 0.00% | 0.00% | 0.00% | 19.25% | 6.21% | 0.00% | 32.30% | 0.00% | 0.00% |
| **Q9SC38** | 25.64% | 0.00% | 0.00% | 0.00% | 30.77% | 14.36% | 0.00% | 29.23% | 0.00% | 0.00% |
| **Q9SC39** | 23.08% | 0.00% | 0.00% | 0.00% | 32.82% | 14.36% | 0.00% | 29.74% | 0.00% | 0.00% |
| **P25890** | 26.32% | 0.00% | 0.00% | 0.00% | 19.84% | 8.50% | 0.00% | 45.34% | 0.00% | 0.00% |
| **C0STY9** | 26.52% | 0.00% | 0.00% | 0.00% | 20.45% | 9.31% | 0.00% | 43.72% | 0.00% | 0.00% |
| **O24296** | 22.46% | 0.00% | 0.00% | 0.00% | 22.03% | 7.63% | 0.00% | 47.88% | 0.00% | 0.00% |
| **P48534** | 36.80% | 0.00% | 0.00% | 0.00% | 14.40% | 11.20% | 0.00% | 37.60% | 0.00% | 0.00% |
| **C7EXK9** | 29.47% | 0.00% | 0.00% | 0.00% | 13.53% | 11.11% | 0.00% | 45.89% | 0.00% | 0.00% |

were retrieved and used for the analysis of proteins. Physico-chemical parameters were calculated using ProtParam analysis, a tool of expasy server and data was represented in Table 2. The analysis provides information about various physic-chemical properties of proteins. Isoelectric point (pI) were calculated which is an important parameter at which the proteins are stable and compact, means with least solubility and zero mobility in an electro-focusing system. Isoelectric point is that pH at which the charge on the protein is zero, no matters that the protein is covered with charge. The evaluated pI value of SOD accession no. P27084 was more than 7 (pI>7), which indicates the antioxidant protein as basic in nature. The pI of other proteins: SOD (P11964, Q02610, Q5DWE8, Q7XHK3, Q6TA12, Q9SC38, Q9SC39), Catalase (P25890, COSTY9), PHGPx (O24296) and APX (P48534, C7EXK9) were less than 7 (pI<7) which specifies acidic character of proteins. This information will be very beneficial for the development of buffer system for the refinement of protein by isoelectric focussing method. The Expasy's Prot Param tool evaluates the extinction coefficients that point out the optical density of a protein, means it provides information about how much amount of light being absorbed by a protein at a certain wavelength. The wavelength of 280 nm is most favoured for proteins because it shows absorbance maxima at this wavelength. These results are basically because of the absorbance of mainly two aromatic amino acids tyrosine (Tyr) and tryptophan (Trp), by the absorbance of cysteine (Cys) (21). The extinction coefficient for the antioxidant proteins SOD (P27084, Q7XHK3, Q6TA12, Q9SC38, Q9SC39), Catalase (P25890, C0STY9), PHGPx (O24296), APX (P48534, C7EXK9) were ranging from 11585 to 87570 M–1 cm–1 in respect of Tyr, Trp and Cys. The high value of extinction coefficient of SOD (P27084, Q7XHK3, Q6TA12), Catalase (P25890, C0STY9) implies that these proteins have high concentration of Tyr, Trp and Cys. This evaluation of extinction coefficients benefits in the study of protein-ligand and protein-protein interaction. The stability of proteins in test tube estimated on the basis of instability index (II) of protein (22). In this method the occurrence of certain dipeptides are unstable which is compared to the stable ones, and provides the weight value of instability. By using weight value, it becomes possible to estimate instability index. Protein with instability index value less than 40 is said to be stable and those with more than 40 are supposed to be unstable. The instability index value ranges from 11.36 to 41.55 for the antioxidant protein of *Pisum sativum.* This analysis concluded that Catalase (P25890 and C0STY9) are unstable proteins, and all the other proteins studied are stable.

Aliphatic index (AI) suggests the thermostability of a protein (23). It is well-defined as the relative volume of a protein occupied by aliphatic side chains (alanine, leucine, isoleucine and valine). The value of aliphatic index for the antioxidant proteins of Pisum sativum varies from the range of 64.20 to 100.41. All the antioxidant proteins are showing very high aliphatic index that means they are stable at a very wide range of temperature. When the summation of hydropathy value is divided by the total number of residues in a protein sequence, it provides the Grand Average of Hydropathy (GRAVY) value (12). Least GRAVY index suggests that the proteins may have better interaction with the water molecules.

The functional characterisation of these antioxidant proteins includes the prediction of disulphide linkage, transmembrane region and motifs. SOSUI server search for a (alpha) helix and predicts the transmembrane region of a protein. It also distinguishes between soluble and membrane proteins. The detailed information about the transmembrane region, length and types were tabulated in Table 4. Only two antioxidant proteins of SOD (Q02610, Q5DWE8) have transmembrane region which indicates that these amino acid sequences contain signal peptide. Other does not have any transmembrane region and lack signal peptides which represent the proteins as soluble proteins. Predicted transmembrane region of Q02610 and Q5DWE8 were represented in Helical Wheel presentation (Figure 1), plot was generated via Pepwheel 32 programme, a part of EMBOSS 6.5.7 suite. To determine the functional linkages,

disulphide bonds are important because the thermostability of a protein depends on it. Identification of the disulphide bond was performed through DiANNA 1.1 server. Cysteine sequence position, distance between the two cysteine residues predicted bond and the predicted connectivity were represented in Table 5. In the antioxidant protein SOD (P27084, Q7XHK3, Q6TA12) and APX (P48534, C7EXK9) no disulphide bonds were predicted for the sequences.

Functional characterisation of antioxidant protein was performed using Prosite database, which provides information about motifs and its position in a protein. Motifs or pattern are the cluster of 10 to 20 amino acid residues in length, have biological function and are conserved during evolution (24). Table 6 contains all the Prosite analysis about the functionality of profile and patterns present in these proteins.

The secondary structural elements were predicted using SOPMA (Self Optimized Prediction Method with Alignment). It is an improved version of SOPM method that accurately predicts 69.5% of amino acid residues for the analysis of the secondary structure mainly three states- alpha helix, beta sheet and coil. Random coils of P11964, Q02610, Q5DWE8, Q7XHK3, P25890, C0STY9, O24296, P48534and C7EXK9 shows dominating role towards the extended strand and alpha helix. In the sequence of P27084 and Q6TA12 the alpha helix dominates the secondary structure. Q9SC38 and Q9SC39 sequence of protein lies in random coil which reveals that the random coil of these two sequences dominates the extended strand and beta turns.

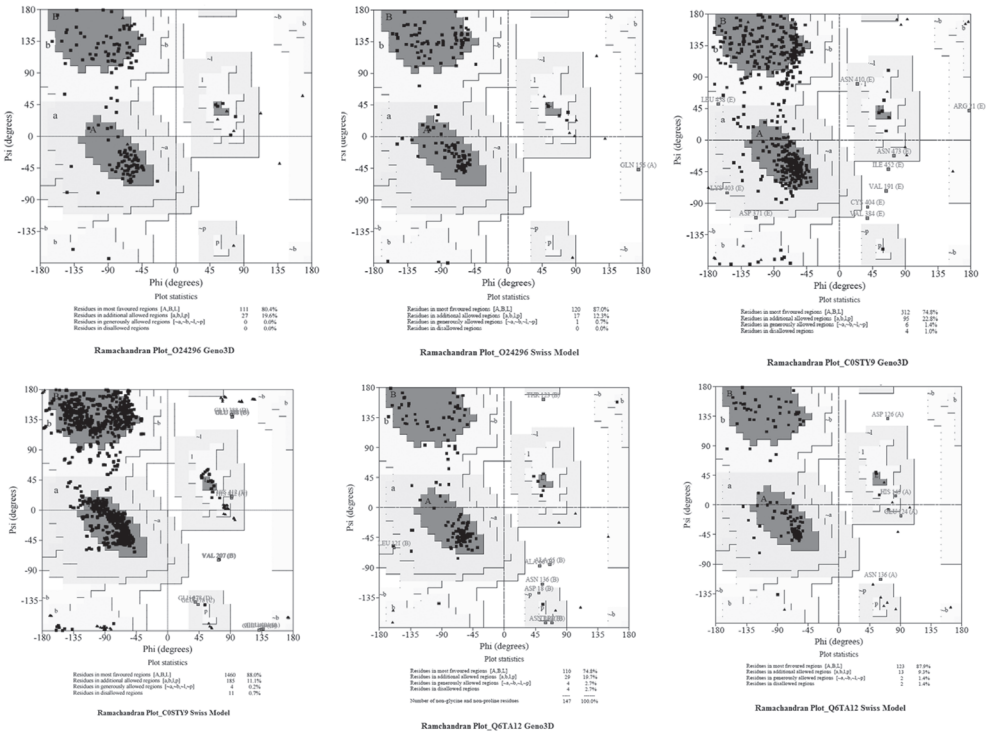


**Fig. 2:** Modeled Structure of antioxidant proteins of Pisum sativum (a) SOD (Q6TA12); (b) Catalase (C0STY9); (c)PHGPx (O24296)

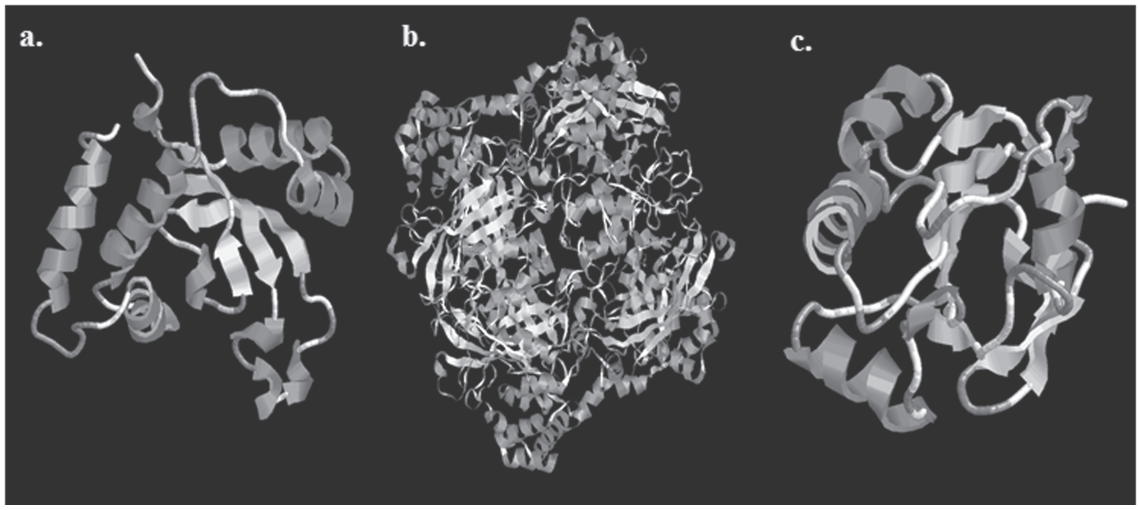


**Fig.3.** Comparative assessment of the protein from Swiss-model and Gene3D with Ramachandran plot showing residues in the most favorable region and disallowed regions.

The tertiary structure of the APX protein, P48534 with PDBe ID: 1APX was present in PDBe (Protein Data Bank Europe). But the experimental three dimensional structures for other antioxidant proteins were not available in any of the structural databases. Q6TA12 Tertiary structure of the protein sequence was generated using Geno3D and Swiss Model by using A chain of 1unf, the tertiary structure of the protein sequence C0STY9 (catalase protein) was modelled using the Swiss-model and Geno3D by using A-chain of PDB 4QOLas a template and for the modelling of the protein sequence O24296 using Swiss-Model program, A-chain of 2P5R PDB structure was selected as a template for modelling of the protein. Then the same protein sequence is modelled using Geno3D server, using B chain of 2P5QPDB structure was selected for homology modelling of the protein. The stereochemistry check for the protein structure modelled through Geno3d and Swiss-Model was performed using Procheck, an integrated tool of Swiss-Model workspace sequence assessment. The constructed models were visualised with the help of Swiss PDB viewer and were shown in figure 2.

The next and most crucial step of homology modelling is the assessment of the generated model. For the evaluation of the final models and examination of their liability of the model's crystal structures, the SAVES (Structure Analysis and Verification Server) were employed. Stereochemical properties as well as constancy of the modelled structures of the protein structure were evaluated by Ramachandran Map analysis in PROCHECK validation package.

The distribution of amino acids around phi and psi angles in Ramachandran Plot was generated by non-proline, non-glycine residues and the comparative result obtained from the Swiss Model and Geno3D server generated models were summarised in table 8.The comparative assessments of Ramachandran Plot were shown in Figure 3. In the Ramachandran plot the amino acid residues were classified on the basis of the regions where they fall in the quadrangle. Red regions in the map shows most allowed regions whereas yellow region shows the allowed region. Only the glycine is symbolized by triangle and others were represented by squares. The result of the protein structure modelled through the Swiss-Model were most acceptable for the SOD (Q6TA12) and Catalase(C0STY9) which have the 87.9% and

88.0% of residues in most favoured region respectively. Only PHGPx (O24296) shows Geno3D modelled structure as most acceptable model in comparison to Swiss-Model with 87.0% of the residues in most favoured region.

**Conclusion**

Antioxidant proteins present in Pisum sativum were selected for this study. Computation analysis for the study of the physio-chemical parameters of the antioxidant proteins were performed by figuring out the isoelectric point (pI), molecular weight, peptide mass, amino acid composition, extinction coefficient, atomic composition instability index, grand average hydropathy. SOSUI server was used to classify and predicts the secondary structure of a transmembrane protein. Other functional characters of protein like disulphide linkage, motifs and patterns were also predicted. Secondary structure was predicted using SOPMA. The modelling of the protein three-dimensional structures was performed using Swiss-Model and Geno3D. Ramachandran plot were generated for validation of modelled protein structures. This analysis will provide additional help for the analysis of crystal structure for further experimentation.

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# Synthesis of biodiesel from chicken waste using egg shell as catalyst

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**Abstact**

Biodiesel (Greek, bio = life + diesel = from Rudolf Diesel) refers to diesel equivalent, processed from biological sources. Biodiesel is otherwise referred as "neat" fuel. It is a fuel derived from a chemical reaction of alcohol (methyl, ethyl, butyl, propyl, isopropyl) and vegetable or animal oils, fats, or greases. These oils or fats are chemically altered in order to use in any diesel engine, with little or no modification. A process called transesterification removes the glycerin component of the oil (which is a triglyceride molecule), resulting in a much thinner, or less viscous product, which stays down to much lower temperatures. It significantly reduces harmful pollutants, as well as carbon dioxide gas and reduces our vehicles contribution to climate change. Current oil and gas reserves are sufficing to last only a few more decades. The scarcity of known petroleum reserves will make renewable energy sources more attractive. To meet the rising energy demand and replace petroleum reserves, fuels, natural gas etc., Biodiesel is in the forefront of alternative technologies. The first record of Biodiesel usage was in 31st August 1937, by the University of Brussels, Belgium who conducted transesterification of vegetable oil using ethanol in order to separate the fatty acids from glycerol by replacing the glycerol with short liner alcohols. More recently Renault and Peugeot have approved the use of biodiesel in some of their own truck engines. In 1991, the European Community (EC) proposed a 90% tax deduction for the use of biofuels, including biodiesel. Now a days each of the plant are producing up to >1.5 billion gallons of fuel per year. The European Union accounted for nearly 89% of all biodiesel production worldwide in 2005. The present study is aimed at Synthesis of Biodiesel from Chicken Waste using Egg Shell as Catalyst.

***Keywords :*** *Biodiesel, Transesterification, Chicken Waste, Egg Shell and Catalyst.*

## Introduction

Biodiesel is a renewable, non-toxic, and biodegradable fuel. There is a great need for biodiesel that does not cause significant harm to environment and does not compete with food supply. It is a well known fact that transport is almost totally dependent on petroleum, diesel fuel, liquefied petroleum gas (LPG), natural gas, fossil based fuels such as gasoline etc (Knothe K. 2008).

In early 1853, Duffyand J. Patrick conducted transesterification of triglycerides in oils. Life of the diesel engine began in 1893 which was published by Dr. Rudolph Diesel. He received a patent in1893 and demonstrated a working engine in 1897. His first model was run using Peanut Oil in Augsburg, Germany on 10th August 1893 after which interest in transesterified vegetable oil and animal fat oil as fuel for internal combustion engines was reported in several countries during the 1920's and later during World War II. The use of vegetable oil as an alternative fuel competing with petroleum has the following advantages, fuel portability, ready availability, renewability, and biodegradability. Since 1980's, biodiesel plants have been opened in many countries such as Europe, United Kingdom, China, Japan, Germany,

Brazil and Italy. These Countries have tested vegetable oil run in buses (Grabosky M and McCormick R. 1998).

There are various other biodiesel sources: almond, soybean, olive, barley, coconut, groundnut, oat, fish oil, tallow, lard, chicken, microalgae, rice, sorghum, tobacco seed wheat, etc. Widespread use of soybean in the USA for food products has led to the source for biodiesel in that country. In Malaysia and Indonesia, palm oil is used in biodiesel source. In Europe, rapeseed is the most common base oil for biodiesel production. In India and Southeast Asia jatropha tree is used as a significant fuel source (Alcantara *et. al.,* 2000).

It has been claimed that Biodiesel gives better lubricity and more complete combustion in increasing the engine energy output.The calorific value of biodiesel is about 37.27 MJ/Kg. The color of biodiesel ranges from golden to dark brown, depending on the production method. The flash point of biodiesel exceeds 130 °C (266 °F). It has a density of ~0.88 g/cm³, which is higher than petrodiesel (~0.85 g/cm³) (Bala, B.K. 2005.)

Biodiesel is a diesel fuel substitute obtained mainly by basic catalytic transesterification of oils and fats. It is composed of fatty acid mono-alkyl esters that are produced from the reaction of low acid number vegetable oil with an alcohol in the presence of a basic catalyst (Fukuda *et. al.,* 2001). Biodiesel is currently produced by the base-catalyzed transmethylation of triglycerides, producing fatty acids methyl esters (FAME). At the end of the reaction, the glycerol rich-phase is separated from the methyl ester layer (Dorado *et. al.,* 2002).

Alcohols are one of the most important raw materials for the production of biodiesel. Alcohols are primary and secondary monohydric aliphatic alcohols comprising of 1, 8 carbon atoms. Methanol and ethanol are the most widely used alcohols in biodiesel production. Other alcohols which are short chained produced in biodiesel are Butanol, Propanol, Isopropanol, tetra-butanol, and branched alcohols. These are however costly. The reaction with triglycerides is quick and it can be easily dissolved with NaOH.

Butanol (also called as butyl alcohol) is a four-carbon alcohol with a formula of C4H9OH, (sometimes represented as BuOH, n-BuOH, i-BuOH). When produced biologically it is called as biobutanol. In blends with diesel or gasoline, Butanol is less likely to separate from the fuel. Biobutanol can be produced by fermentation of bacterium *Clostridium acetobutylic*um, also known as the Weizmann organism, or *Clostridium beijerinckii*. Biobutanol can also be made using *Ralstonia eutropha* H16. This process requires the use of an electro-bioreactor and the input of carbon dioxide and electricity. Additionally, Butanol production from biomass and agricultural byproducts could be more efficient (i.e. unit engine motive power delivered per unit solar energy consumed) than ethanol and methanol production. In 2012, Researchers developed a method for storing electrical energy as chemical energy in higher alcohols (including Butanol). These alcohols can be used as liquid transportation fuels. In the late 2012, a new discovery made the alternative fuel Butanol more attractive to the Biofuel industry. Scientist Hao Feng found a method that could significantly reduce the cost of the energy involved in making Butanol. Also in late 2012, using systems metabolic engineering, a Korean Research Team at the former Korea Advanced Institute of Science and Technology has succeeded in demonstrating an optimized process to increase Butanol production by generating an engineered bacterium (Demirbas, A. 2002).

Chicken fat is high in linoleic acids, the beneficial omega 6 fatty acid. Linoleic acid levels are between 17.8 – 22.9 %, which was used as pet food and also for biodiesel production (Heena Sharma *et.al.,* 2013). The fat tissues are cooked and the fat is released by temperature and cell rupture (Mata T.M. *et.al.,* 2011). In alternative process the temperature is kept low and the fat is released principally through mechanical rupture of the cell (Vivian Feddern *et.al.,* 2011).

Every year enormous amount of waste shells are disposed into the landfills. Waste shells such as egg shells or sea shells are rarely used to produce practical products and environmentally benign solid catalyst for the biodiesel synthesis (Thi Thi Win, 2015). Biodiesel synthesis that employs waste shell is a viable fuel alternative when compared to petroleum-derived diesel. Biodiesel synthesis is more advantageous in the aspect that it is much faster in its production rates as compared to the rate of formation of natural fossil fuel production. Waste egg shells possess Calcium Carbonate (CaCO3) which acts as a catalyst (Israa M. Rashid *et.al.,* 2015).

**Materials and Methodology**

Transesterification is widely used to reduce fat oil viscosity. The transesterification was carried using a continuous magnetic stirrer. 200 ml of chicken oil was taken in a 500 ml flat bottom flask equipped and with a reflux condenser was stirred at 600 rpm for all test runs. The oil was heated at 65°C for 5 minutes in a heating water bath to evaporate water and other volatile impurities. A mixture of oil and catalyst were stirred with a magnetic stirrer at about 600 rpm. A designated amount of butanol was added into the reactor. Each cycle was allowed to continue at 65ºC. After finishing the reaction, the mixture was allowed to cool down and was centrifuged to separate the catalyst. The resulted catalyst was treated with butanol to reuse or regenerate. The mixture was filtered to remove catalyst absolutely. After this, the mixture was settled in a separating funnel for 10 minutes to separate the layers clearly. The upper layer consisted of butyl esters and unconverted triglyceride. The lower layer contained glycerol, excess butanol and soap formed during the reaction and also possibly some entrained butyl esters. The present study is aimed at Synthesis of Biodiesel from Chicken Waste using Egg Shell as Catalyst. Estimation of Cholesterol, Saponification Value, Iodine Value, Acid Value of Fats, Alkali Catalyzed Transesterification and FTIR Analysis was carried out using Standard Protocols.

**Results and Discussion**

**Chicken waste fat from skin**

Animal fat wastes can be used for the extraction of biodiesel by alkali-catalyzed process under mild conditions. A mixture of chicken fat residue is used as feedstock for biodiesel production. Chicken fats are highly viscous and mostly in solid form at ambient temperatures because of their high content of saturated fatty acids. They are readily available from slaughter houses and butcher shops (Figure 1).

**Figure 1:** Chicken Skin Fat residue as feedstock for biodiesel production

**Oil Extraction**

The oil was extracted from chicken waste (skin) fat by heating process. Thawed chicken byproducts (mixture of skin and chicken waste parts) were placed on to a container and rinsed with distilled water 2 - 3 times, to remove impurities. The liquid was drained and the chicken waste was heated at 60 °C for 10 minutes. The liquid vapourizied and the oil was extracted (Figure 2). The Oil was filtered by filtrate paper and was stored in a separate flask.

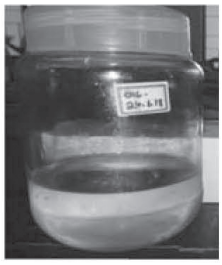

**Figure 2 :** Oil extracted from thawed chicken waste by heating process

**Catalyst Preparation**

The waste eggshells were used as a precursor of CaO in this study. To remove all unwanted materials adhered to the surface; they were washed thoroughly with tap water and rinsed several times with deionized water. After this procedure the eggshells were sun dried. The dry eggshells were crushed and sieved in china dish set. The dried powder was calcined in a micro-oven at 380°C for 1 hour (Figures 3 & 4).

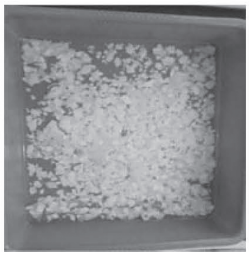

**Figure 3**: Chicken egg shells

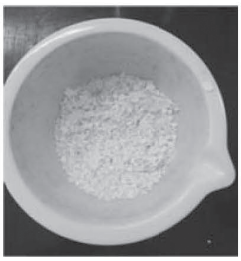

**Figure 4**: Dried egg shells calcined at 380 °C for 1 hour

**Catalyst Characterization**

The waste shells were examined by using a Scanning Electron Microscope (SEM) to emphasize the morphology of the catalyst. The crystalline structure of the catalyst was analyzed by X-ray Diffraction (XRD). Scanning Electron Microscope (SEM) was used to observe the microstructure of eggshell powders calcined at different temperatures (110 °C, 200 °C, and 380 °C.) and different periods of time (10 minutes, 30 minutes, 60 minutes.) The eggshell before calcination had macro pores and an irregular crystal structure, SEM images showed that the structure of eggshell changed with calcination temperatures and time (Figures 5 - 7).

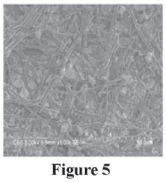

Figure 5

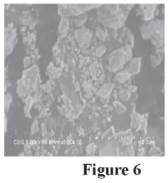

Figure 6

**Figure 5 :** SEM image of egg shell before Calcination
**Figure 6 :** SEM image of egg shell after Calcination

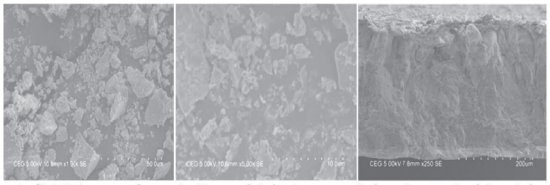

**Figure 7 :** SEM Image of egg shell, particle's shape and size changed with calcination with different temperatures and time

**X-ray Diffraction**

The XRD pattern of egg shell catalyst at different calcination temperatures and periods of times showed phase change of calcium compounds CaO. The diffraction peaks obtained at 24.3, 38.2, 47.1, 56.8 were the characterizations of CaO (Figure 8).

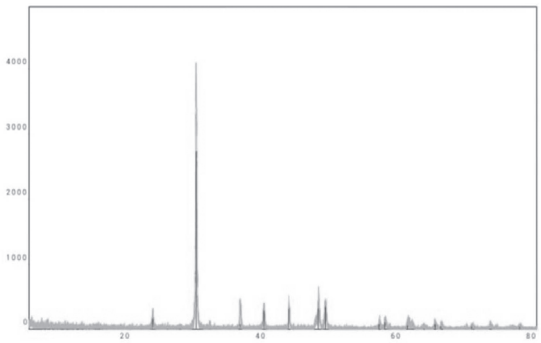


**Figure 8 :** X-ray Diffraction Pattern of the Eggshells

**Transesterification**

Transesterification also called alcoholysis is the reaction of a fat or oil triglyceride with an alcohol to form esters and glycerol. A catalyst is usually used to improve the reaction rate and yield. Because the reaction is reversible, excess alcohol is used to shift the equilibrium to the product side. The biodiesel reaction requires a catalyst such as calcium carbonate to split the oil molecules and an alcohol (butanol or ethanol) to combine with the separated esters. The main byproduct is glyceride. The process reduces the viscosity of the end product (Figure 9).

**Figure 9 :** Transesterified Oil

## FT- IR Analysis

The Transesterified Fuel was observed between the Spectra Range 600 – 1500 Wavelength where 87% of Biodiesel is similar to Normal Diesel (Figure 10).

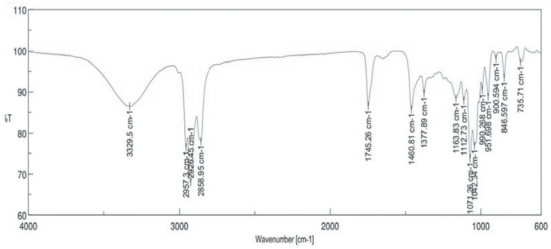


**Figure 10** : FTIR Analysis of Transesterified Oil - Biodiesel

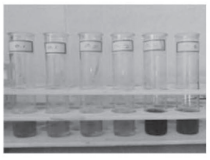

**Figure 11 : Cholestrol estimation test by Zak's method**

**Figure 12: Estimation of Iodine Value**

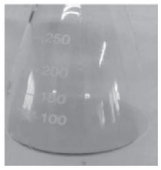

**Figure 13: Acid value estimation, pink color observed as end point**

## Cholesterol estimation

Cholestrol reacts with ferric chloride acetic acid in the presence of concentrated sulphuric acid to give pink colour due to charring of cholestrol by sulphuric acid. The colour was read at 560nm (Figure 11). 1ml of the Oil concentration contained 17 mg of cholestrol.

## Estimation of Saponification Value

2 g of oil was refluxed with an excess amount of alcoholic KoH. After saponification, the KoH was estimated to be 78.54 mg.by titrating it against hydrochloric acid. Fatty acids in the glycerides of the oil were hydrolysed by an alkali (hydrochloric acid) This value is useful for a comparitive study of the fatty acid chain length in oil.

**Figure 14 : Burning of Spirit Lamp using the produced Biodiesel**

**Figure 15 : Biodiesel in Cosmetics**

**Figure 16 : Biodiesel Motor Testing**

**Estimation of Iodine Value**

Iodine value is a measure of the degree of unsaturated oil. Iodine value is useful for studying oxidative rancidity of oils. The measure of iodine absorbed by an oil, gives the degree of unsaturation ( Figure 12).

**Estimation of Acid Value**

KoH is standardized, so that it can be used for the estimation of acid value of the oil sample. The normality of standardized KoH is 0.073. The acid value for the following Chicken waste fat oil was estimated to be 5.57 (Figure 13). The acid value in milligrams of KoH to neutralize the free fatty acid present is 4.971 g of fat.

**Biodiesel testing**

The purified form of biodiesel was tested as such by burning a spirit lamp using it as a fuel (**Figure 14**).

**Biodiesel in Cosmetics**

The purified crude oil was taken in a clean watch glass. To 2 ml of the essential body oil, 0.8 ml of chicken fat oil was added. It was then used as body oil. A scoop of petroleum Vaseline was added with 1 ml of fat oil in a clean watch glass. It was mixed thoroughly until it blended well with the Vaseline gel. It was further used as a skin lotion (Figure 15).

**Biodiesel Efficacy**

1-2 liters of transesterified biodiesel was transferred into a diesel engine motor. Efficacy of biofuel test was successful by witnessing the running of the motor (Figure 16).

**Conclusion**

Animal fats have great potential as feedstock's for biofuel segments, because they are not commodities, having a lower market value. Over the last years, meat production has increased significantly. The catalyst derived from chicken eggshell had excellent activity in heterogeneous transesterification of chicken fat oil for biodiesel production. This catalyst contains CaO which is converted after calcination at temperatures 360°C for 1hr. The method of reusing chicken eggshell wastes to prepare catalyst could recycle the waste, minimizing contaminants, reducing the cost of catalyst, and making the catalyst environmentally friendly. Here chicken eggshell heterogeneous catalyst showed high activity and thus this low- cost catalyst could be used in a large- scale industrial production of biodiesel, making the process cheap and ecologically benign. Dry purifications makes the process environment friendly and substantial reduction in the total time of production. Biodiesel an excellent alternative of diesel fuel is renewable, biodegradable, and non-toxic. It refers to a vegetable oil or animal fat based diesel fuel consisting of long chain alkyl esters. Oil extracted from chicken waste meat fat was converted into biodiesel. Chicken waste egg shells were used as catalyst, and they were calcined under 380 °C for 1 hour. It was transesterified and the conversation percentage was found to be 87%. The obtained crude biodiesel was washed with water to remove remaining catalyst and impurities. It was allowed to dry to remove the water particles. The same procedure was repeated to study the effect of oil in different parameters such as Cholesterol Test, Saponification, Iodine Value and Acid Value to note conversion of fatty acids into biodiesel. It was then subjected to analysis such as XRD, SEM and FT- IR to test its purity.Thus the present study indicates that Biodiesel can be easily produced as a promising alternative fuel and thereby also helping in reducing pollution and health hazards to our society. Let's work towards a "**A BioGreen Tomorrow".**

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# Production of bio-diesel from non-edible dried fruits of *Lagerstroemia speciosa*

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**Abstract**

Rapid urbanization and increase in population have evoked tremendous attention for biofuelsproduction to combatshortage of fuels, environmental concerns, foreign exchange savingsand socioeconomic issues.In recent years biodiesel production from agro-industrial feedstocks such as waste vegetable oil, animal fat, grease, non-edible fruit oils etc.,acquired prominent place to fulfil the gap between production and demand. The present investigation has been undertaken to explore a noveland environmentally friendly process for developing biodiesel production technology by subjecting dried fruits of *Lagerstroemia speciosa* to mild ultrasonication at 33KHz for 20 min at 35±2ºCfor obtaining high lipid yield, precursor for the production of biodiesel by transesterification.The biodiesel compounds 2,4-di-tert-butylphenol, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid (Z, Z) methyl ester, 9-octadecenoic acid methyl ester, methyl stearate, cis-11,14-eicosadienoic acid methyl ester, 18-methylnonadecanoate were recognized as the main compounds in GC-MS analysis.

**Keywords:**Biodiesel, *Lagerstroemia speciosa*, dried fruits, ultrasonication, transesterification

## Introduction

Fossil fuels such as petroleum, diesel, gasoline and liquefied petroleum gas occupied a prominent place in transportation industry (1).In order to meet the transportation demands of growing population, natural resources are explored extensively.Global fossil fuels consumption expected to decline1000 million tonnes from 4000 million tonnesby 2050 due to the diminution of fossilfuel resources (2).Over time, exponential utilization of fossil fuels has generated an imbalance in the carbon cycle, dramatically increasing greenhouse gas and contributing to climate change (3).To combatshortage of fuels, environmental concerns, foreign exchange savingsand socioeconomic issues related to the rural sectors of all countries in the world has urged a research interest for biofuels such as bioethanol, biobutanol, biodiesel, green diesel, bio methanol, dimethyl ether, bio-oil production (3-10).In recent years biodiesel production has evoked tremendous attentiondue to the advantage of environmental compatibility, biodegradableand alternativeto petroleum fossil fuel (11).Biodiesel is a non-petroleum based alternative diesel fuel comprised of monoalkyl esters of long-chain fatty acids produced by transesterification of oils (11-13).Due to excess consumption of biodiesel, the gap between production and demand is widening. To fulfil the gap, with the advent of science and technology many researchers adopted new methods to explore various alternative agro-industrial waste oils for the production of biodiesel, such as soybean, rapeseed, coconut, rice bran, barley, wheat, peanut, corn, olive, sunflower, palm, jatropha and neem oils(14, 15).Of these oils, non-edible fruit oils dominate the biodiesel sector. However, utilization of non-edible dried fruits of *Lagerstroemia speciosa* for the production of

biodiesel has not been reported. The plant *Lagerstroemia speciosa* belongs to the family *Lythraceae* of dicotyledons. This plant is commonly called as "Pride of India" It is a small to medium-sized tree growing to 20 m (66 ft) tall, with smooth and flaky bark, deciduousoval to elliptic leaves that are 8-15 cm (3.1-5.9 in) long and 3-7 cm (1.2-2.8 in) broad with an acute apex.The flowers are produced in erect panicles of 20-40 cm (7.9-15.7 in) long, each flower has six white to purple petals of 2-3.5 cm (0.79-1.38 in) long (16,17).The present research will be focusing on extraction of oils from non-edible dried fruits of *Lagerstroemia speciosa* for the production of biodiesel.

## Materials and Methods

### Experimental Chemicals

All chemicals and reagents used in this research were of analytical grade and are mostlypurchased from Sigma, USA and Hi-media, Mumbai.

### Plant Collection and Preparation of Powder for Lipid Extraction

Dried fruits of *Lagerstroemia speciosa* used for this study was collected in the month of November, 2018,at the location of 13.043443 N, 77.591154 E,Bruhat Bengaluru Mahanagara Palike (BBMP) park, Hebbal, Bengalore, Karnataka, India and authenticated at Regional Ayurveda Research Institute for Metabolic Disorders, Bangalore, by Dr. V. Rama Rao with an accession number, RRCBI-3933(Fig-1).The sample was collected in a sterile plastic container and it was brought to the laboratory for further processing (11).

The dried fruits were washed for 2-3 times with tap water to remove the surface debris and then dried in an oven at 40°C for 24 h(18). The dried fruits were milled to a coarse powder and stored in air tight container for oil extraction.

**Extraction of Lipid from Powder :** The total lipid content was extracted by modified Bligh and Dyer method(19).Experiment was conductedin

**Fig-1:** Fruits of *Lagerstroemia speciosa*

two sets(A and B).In a 250 ml conical flask 10 g of dried powderand two-fold of 2M HCL were added and the mixture was vortexed for 24 h. After the 24h set A was subjected to sonication (GTSonic-GT-1730QTS)at 33KHz for 20 min at 35±2ºC. During the treatment process, the temperature was maintained at 35±2ºC by adding ice cubes(20). The set B was considered as control. Both the sets were subjected to centrifugation at 5000rpm for 10 min (Remi C24 Plus). After centrifugation the supernatant from set A and pellet suspension from set B was transferred in to a 100 ml conical flask containing 4ml of distilled water, 20ml methanol, and 10ml of chloroform and vortexed for 15min at room temperature. The chloroform layer was separated by centrifuging at 5000rpm for 10 min. After centrifugation, the chloroform phase was evaporated with rotary vapor and the residue was preserved for transesterification.

**Biodiesel Generation by Transesterification of the Lipids :** Transesterification was carried out in two sets in a 250ml glass beaker equipped with a magnetic stirrer. 10ml of the lipid was taken in separate beakers for set A and set B and heated

up to 50°C on hotplate (DLAB MS- H280- Pro). For both the sets 60ml of sodium methoxide was added and stirred vigorously for 1 h on a magnetic stirrer(21). The mixture was transferred to a separating funnel and glycerol was allowed to settle and separate for 24 h. After draining the glycerol, methyl esters present in the upper layer was collected and analyzed using Gas Chromatograph-Mass Spectrometer.

**Analysis of Fatty Acid Methyl Esters :** Biodiesel producedfrom dried non-edible fruits of *Lagerstroemia speciosa*was analysed by using mass spectrometer (ShimadzuGCMS-QP2010SE) with two narrow-bore capillary columns, coupled to agas chromatograph (Shimadzu GC-QP2010). The GC column used was fused with silica capillary column(QP2010, 30m × 250 μm, film thickness 0.25 μm). The pressure ofthe carrier gas (helium) was 72.6 kPa Psi at the initial oven temperaturewith flow rate of 6.6 ml/min. All standards and samples were injectedin split mode (split/column flow rate 1.20ml/min). The injector temperaturewas 250°C; the column oven temperature was 60°C, rose to 280°Cand total run time was 40 min. The mass spectrometer wasoperated in the electron impact (EI) mode at 82 eV in the scan range of35-500 m/z. The temperature of the transfer line and of the ion sourcewas set to a value of 200 and 280°Crespectively. The injection samplevolume was 8.0 ìl.Peak identification of an oil was performed bycomparison with retention times of standards and the mass spectraobtained compared with those available in the Wiley and NIST libraries(Wiley Registry TM, 8th Edition Mass Spectral Library and the NIST 08Mass Spectral Library (NIST/EPA/NIH) 2017 version) with an acceptancecriterion of a match above a critical factor of 80%.(11,22).

**Results and Discussion**

In biodiesel producedfrom sonicated dried non edible fruits of *Lagerstroemia speciosa*,2,4-di-tert-butylphenol, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid (Z, Z)-methyl ester, 9-octadecenoic acid methyl ester, methyl stearate, cis-11,14-eicosadienoic acid methyl ester, methyl 18-methylnonadecanoate were recognized as the main compounds in GC-MS analysis (Table-1 and Fig-2 and 3). In non-sonicateddried non-edible fruits of *Lagerstroemia speciosa*,hexadecanoic acid methyl ester, 9,12-octadecadienoic acid (Z, Z)-methyl ester, 9-octadecenoic acid methyl ester andmethyl stearate were recognized as main compounds (Table 2 and Fig 4 and 5).According to NIST17.lib library and previous reports of Dwivedi *et al,*9,12-octadecadienoic acid (Z, Z)-methyl ester was considered as one of the chief biodiesel compound and same was the major constituent with 83.23% of peak area (Fig-6), with retention time of 21.4, 85.18% of peak area with a retention time of 21.4 for both sonicated and non-sonicated samples(23).

**Table 1:** GC-MS results of sonicated dried non-edible fruits oils of *Lagerstroemia speciosa*

| S.No | Peak Value | Compound name |
|---|---|---|
| 1 | 13.481 | 2,4-di-tert-butylphenol |
| 2 | 18.782 | Hexadecanoic acid methyl ester |
| 3 | 21.443 | 9,12-Octadecadienoic acid (Z, Z)-methyl ester |
| 4 | 21.527 | 9-Octadecenoic acid methyl ester |
| 5 | 21.932 | Methyl stearate |
| 6 | 24.458 | cis-11,14-Eicosadienoic acid methyl ester |
| 7 | 24.924 | Methyl 18-methylnonadecanoate |

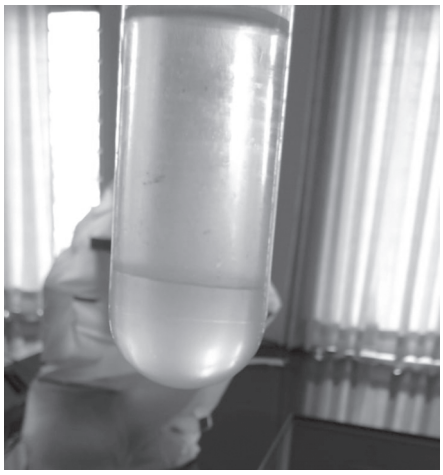

**Fig-2:** Development of organic layer in sonicated sample

**Table 2:** GC-MS results of Non-sonicated dried non-edible fruits oils of *Lagerstroemia speciosa*

| S.No | Peak Value | Compound name |
|---|---|---|
| 1 | 18.780 | Hexadecanoic acid methyl ester |
| 2 | 21.438 | 9,12-Octadecadienoic acid (Z, Z)-methyl ester |
| 3 | 21..526 | 9-Octadecenoic acid methyl ester |
| 4 | 21.930 | Methyl stearate |

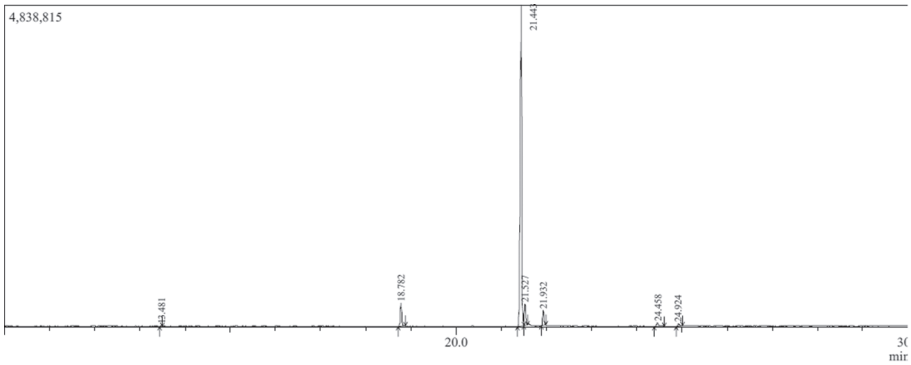


**Fig-3**: GC-MS image of Sonicated sample

**Fig-4:** Development of organic layer in non-sonicated sample

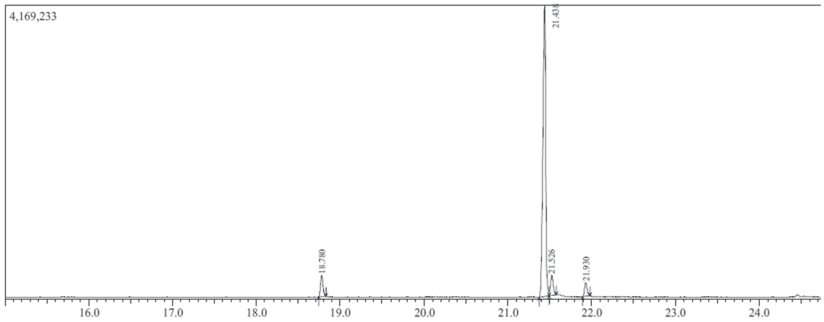


**Fig-5:** GC-MS image of Non-Sonicated sample.

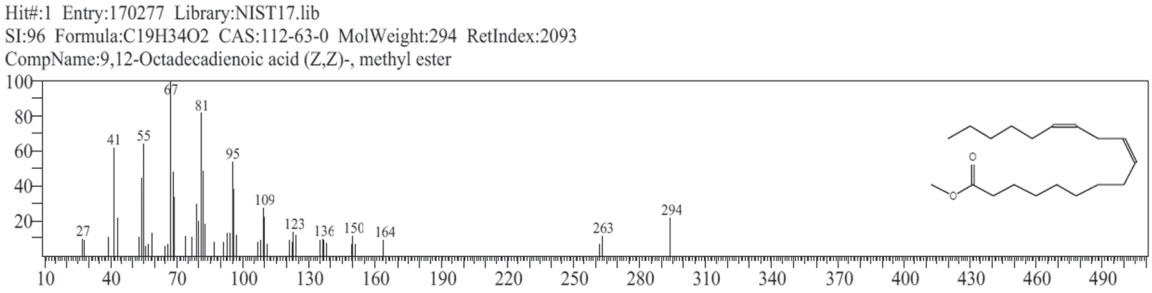


**Fig-4:** Image of the Compound Structure 9,12-Octadecadienoic acid (Z, Z)-, methyl ester.

## Conclusion

Biodiesel production has evoked tremendous attention due to the advantage of environmental compatibility, biodegradableand alternativeto petroleumfossil fuel. In order to fulfil the gap between production and demand various techniques adopted for obtaining high yield of biodiesel from the agro-industrial residues, which are considered as environmental pollutants. Based on our research we are suggesting application of ultrasonication will also help in generating high yield of biofuel producing compounds from dried waste. This technique may be applicable for large scale production of eco-friendly biofuels by exploring other substrates.

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# Prevalence of hepatitis B virus genotypes and sub-genotypes in north and east regions of India: DNA sequencing methodology

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**Abstract**

*Introduction:* Hepatitis B virus (HBV) is a highly prevalent infecting virus among liver-related diseases. The genetic distribution and identification of HBV genotypes and sub-genotypes represented a challenge to control the spread of infection. To find out molecular prevalence, the present methodology was carried out for the distribution pattern of HBV genotypes and sub-genotypes in north and east regions of India. *Methods:*A total of 67 HBV DNA positive subjects were studied. At first, the DNA samples for HBV positive cases were screened by Real-time PCR, and then the selected region of HBV *polymerase* gene was amplified for the sequence analysis to determine genotypes and sub-genotypes. *Results:*The prevalent genotype found was the genotype D (62.68 %), followed by genotype A (29.85 %), and genotype C (7.46 %). Sub-genotype C1 was identified in the east region only. The frequency of sub-genotype A1 was higher in the north region (n = 13, 30.95 %) followed by east region (n = 7, 28 %). HBV sub-genotype D1 was found to be predominant in 15 (35.71 %) subjects followed by sub-genotypes D2 in 10 (23.80 %) subjects from the north region. HBV sub-genotype D2 was found to be predominant in 8 (32 %) subjects from the east region. *Conclusions:*In conclusion, the method clearly demonstrates the high prevalence of sub-genotypes D1, D2, A1 in this region. Also, the identification of the sub-genotype C1 in the east region emphasizes the high transmission infection risk and transmission route towards other regions of India.

**Keywords:** Epidemiology, Phylogenetic analysis, Polymerase chain reaction, Sequencing.

## Introduction

Hepatitis B virus (HBV), belongs to family *Hepadnaviridae*, is a partially double-stranded DNA virus and bears approximately 3.2 *kb* nucleotides. It has four partially overlapped open reading frames encoding for hepatitis B surface proteins, core peptide, X peptide, and DNA-*polymerase* enzyme. HBV genome, having sequence heterogeneity, replicates by reverse transcription using a *polymerase* that lacks proof-reading activity (1). HBV has been circulated 400 million people worldwide (2), with a high prevalence in Asia and Africa. HBV is the most common cause of chronic hepatitis, Hepatocellular carcinoma (HCC), cirrhosis and liver failure (3 and 4). Being a global health problem, some patients affected mildly (2), while other patients developed HCC, cirrhosis and, death from chronic Hepatitis B Virus (5).

Full-length genomes comparisons from different geographical regions classified the HBV into ten genotypes from A to J, based on nucleotide differences by more than 7.5% and further segregate into sub-genotypes, differ from each other by 4 to 7.5% (6). It has been reported that different genotypes and sub-genotypes show different geographical distribution, disease

progression, the response on antiviral treatment, and prognosis, but the mechanisms of different pathogenicity of HBV genotypes are unknown. More than 30 sub-genotypes belonging to HBV genotypes have been determined. Genotypes A, B, C, D, F and I are divided into sub-genotypes, whereas no sub-genotypes have been defined for genotype E, G, and H (7). Genotype D is most common and consist 9 sub-genotypes, where D1 to D3 appear worldwide while D4 to D9 have limited distribution (8). Genotype Ae has been defined in Europe and Aa in Asia along with Africa (9). For the classification of the genotype C, Sub-genotype C1 is found in South-east Asian countries like Thailand, Myanmar, Vietnam, C2 in East Asian countries like Japan, Korea, China (10), C3 in Oceania comprising strains and C4 in Aborigines from Australia (11).

Although HBV viral load is necessary to identify the risk for HCC, cirrhosis, and death in hepatitis infection (5), it is also proposed that viral load is essential, but not sufficient (12). Therefore, other viral markers are required for diagnosis and treatment of the patient. The viral load, circulating genotype and specific risk behavior in a region can help the information available in the country to support the development of specific prevention strategies for the exposed population. Very limited scientific studies are available on HBV genotyping in Indian subcontinents (13). The other challenge for molecular epidemiology in India is to determine the reliable target region to identify genotypes and sub-genotypes with the simple and one experimental process. In this study, we have carried out to find the prevalence and distribution of HBV genotypes with sub-genotypes among the HBV infected subjects in the Indian region. It is also covered the aspects of using simple method to identify molecular epidemiology and distribution pattern of HBV genotypes and sub-genotypes in north and east regions of India.

## Materials and Methods

### Ethical statement

This study was supported by Kumaun University, Nainital and CORE Diagnostics, Gurugram and approved by its ethics committee. The informed consent form was obtained from all the subjects or patients included

### Study subjects

A total of 83 infected subjects with hepatitis B were enrolled in this study. The study subjects were covering the patients of the northern region (n = 53), from the Delhi (New Delhi = 50), and Uttar Pradesh (Ghaziabad = 3) and the eastern region (n = 30), from the Bengal (Kolkata = 6), Bihar (Patna = 10), Orissa (Cuttack = 2, Ling-raj = 1), the eastern country region; Bangladesh (Dhaka = 9) and Nepal (Kathmandu = 2) that were diagnosed with HBV DNA infection. North-east states of India were not the part of our study. The subjects that recruited between June 2017 and December 2017 were part of our study. Blood was collected from the subjects for HBV diagnosis and processed in the laboratory. The samples were tested by commercial Real-time PCR (QIAGEN, Germany) to determine the HBV DNA infection.

The mean age of the patients included in this study was 32.02 ± 15.21 years. Plasma was separated from blood and stored at -20°C until the extraction was done. After that, samples were tested for HBV Genotyping or sub-genotyping by using specified primers.

### Viral DNA extraction and HBV DNA confirmation

Viral DNA was extracted from 500 µl of plasma samples by QIAamp® DSP virus extraction procedure using silica column-based technology (QIAGEN, Germany). HBV DNA was detected and quantified by artus® HBV RG PCR kit (QIAGEN, Germany) according to the instruction of manufacture on the Real-time PCR system (Rotor-Gene Q, QIAGEN, Germany).

### Genotyping with HBV polymerase gene region

Primers used were; forward: 5'- TCGTGG TGGACTTCTCTCAATT-3' and reverse; 5'-CGTTGACAGACTTTCCAATCAAT- 3' for the partial HBV polymerase gene region (14). The composition of 30 µl reaction volumes of PCR master mix was contained 10X PCR buffer, 50

mM $MgCl_2$, 10 mM each of the four dNTPs, 10 μM of each primer with a final concentration of 0.33 μM and 5U of *Taq* DNA polymerase. The temperature parameters were; $95^0$C for 15 minutes, followed to 45 PCR cycles at $95^0$C for 45 seconds, $56^0$C for 45 seconds, and $72^0$C for 45 seconds. Around 730 *bp*products recovered on agarose gel on amplification with primers. After that, clear and strong bands of PCR positive samples were selected for direct sequencing. The samples that shown weak bands or did not produce satisfactory sequence were excluded from the study.

**Sequencing of the amplified product**

The composite PCR products were purified using spin column-based purification kit according to the manufacture instruction, and the purified product was measured by Nanodrop spectrophotometer. The Reverse primer was used as a sequencing primer for all the samples. Amplified PCR products were directly sequenced in the ABI 3500xL Genetic analyzer (Applied Biosystem, USA) Instrument, using the Bigdye terminator (Version 3.1) cycle sequencing kit. For the sequencing, thermal cycling, conditions used were 20 seconds on $95^0$C, 25 seconds on $50^0$C for 35 cycles, and $60^0$C for 2 minutes. Data collection and assembly were done by 3500xL Genetic Analyzer data collection and sequencing analysis software (Version 1.0 and 5.4).

**Sequence analysis**

Sequences were analyzed using KB™ Basecalling (Version 1.4.1.8) sequence analysis software. Sequences received were edited in Chromas software (Version 2.6.4.0) with the comparison of known sequences from the Genbank database, and saved as FASTA file format. Multiple sequence alignments and phylogenetic analysis were done using reference sequences available from Genbank.

**Genotyping & Sub-genotyping determination**

Obtained sequences were aligned with published sequences from the GenBank database with known HBV genotypes and sub-genotypes (15, 16 and 17). Multiple sequence alignment was performed by using CLC Sequence Viewer, Version 6.1 (CLC, Denmark) software. HBV Genotype and sub-genotype were determined by phylogenetic analysis in CLC Sequence Viewer, Version 6.1 using the neighbor-joining method with a bootstrap analysis of 1000 replicates. This analysis is based on partial reverse transcriptase regions of HBV polymerase sequence. Genotype and sub-genotype of HBV were also determined by the Basic Local Alignment Search Tool (BLASTN) program, available in http://www.ncbi.nih.gov/ projects with reference to viral nucleotide sequences. That was done by BLAST of the query sequence with the known set of sequence (18).

**Accession numbers on submission**

After analyzing the 67 HBV polymerase gene sequence**s,** we submitted our sequenced to GenBank, BioSample submission as SUB4043488, and we obtained accessions number from SAMN09237416 to SAMN09237482 for our ascending order sequence.

**Statistical analysis**

Statistical analysis was done using Microsoft Excel. Demographic Variables were measured as mean ± SD. Comparisons for categorical variables were analyzed using the *Fisher's exact* test and the difference for a *p*-value of <0.05 was considered statistically significant.

The informed consent form was obtained from all the subjects or patients included, and the study approved by ethics committee of CORE diagnostics, Gurugram with permission of Kumaun University Nainital.

**Results**

The study subjects that included to defined genotypes and sub-genotypes were from north and east regions of the Indian subcontinent. Subjects from the north region identified (n = 42) were of Delhi (New Delhi = 40), and U.P. (Ghaziabad = 2), Subjects from the Bengal (Kolkata = 5), Bihar (Patna = 10), Orissa (Cuttack

= 2, Ling-raj = 1), and the adjacent country; Bangladesh (Dhaka = 6) and Nepal (Kathmandu = 1) defined the east region (n = 25) results.

The limit of quantification with the Real-time PCR was the 10.5 IU/ml for the HBV DNA viral load. The HBV DNA levels measured using TaqMan Real-time PCR (QIAGEN, Germany), for the samples used in sequencing study were the ranged from 500 IU/ml to 2 × $10^7$ IU/ml. When amplified the positive sample with specific primers, PCR product of approximately 730 *bp* was revealed on agarose gel electrophoresis (Figure 1), of the viral load ranged from 608 IU/ml (limit of detection) to 2 × $10^7$ IU/ml. Furthermore, 67 samples were successfully sequenced and analyzed.

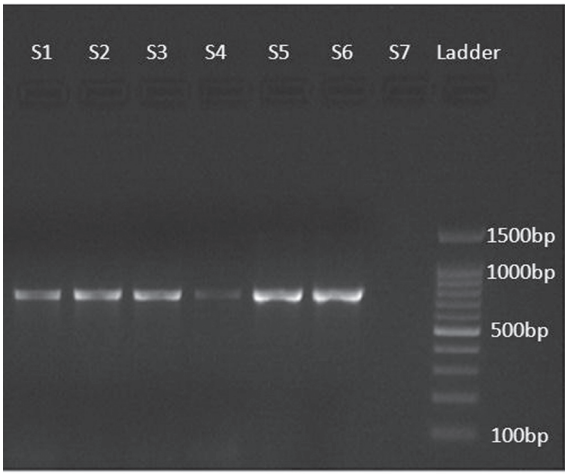


**Figure 1**: Agarose gel electrophoresis showing PCR positive samples in the lane S1 to S6 (band size approximately 730 *bp*) for HBV partial polymerase gene sequence and a negative control in lane S7, followed by ladder 100*bp*.

**HBV genotypes**

Total of three HBV genotypes A, C, and D was found in this population. Of the 67 samples, 42 were detected as genotype D, found to be the predominant circulating genotype (62.68 %). 20 were the genotype A (29.85 %) and 5 were the genotype C (7.46 %). The observed sequences [GenBank: Biosampleaccessions number; SAMN09237416 - SAMN09237482] were aligned with the reference sequences available from GenBank for all known HBV genotypes and determined by constructing a phylogenetic tree. The genotypes of observed sequences were also identified by the genotyping tool (NCBI) with same results.

**Distribution of HBV genotypes within the region**

Distinct patterns of the HBV genotype identified in the study populations from the regions are shown in Table 1. Among the 42 subjects from the north region, HBV genotype D was the predominant genotype, identified in 29 (69.04%) subjects compared to genotypes A identified in 13 (30.95%) subjects ($p < 0.00$). Among the 25 subjects from the east region, HBV genotype D was predominantly identified in 13 (52%) subjects. The other genotypes A and C were identified in 7 (28%) and 5 (20%) subjects, respectively ($p < 0.00$).

**HBV sub-genotypes**

Among the 20 genotype A sequences, all identified as sub-genotype A1 (Figure 2). HBV genotype D sequences were clustered with sub-genotypes D1 (n = 16, 38.09%), D2 (n = 19, 45.23%), D3 (n = 4, 9.52%) and D5 (n = 4, 9.52%) (Figure 3). All 5 HBV genotype C sequences were identified as sub-genotype C1 (Figure 4).

**Distribution of HBV sub-genotypes within the region**

The distribution of HBV sub-genotypes with their frequency are shown in Table 1. Sub-genotype C1 was identified only in the east region. The frequency of sub-genotype A1 was higher in the north region (n = 13, 30.95%) followed by east region (n = 7, 28%). Sub-genotypes D1, D2, D3 and D5 were identified in 15 (35.71%), 10 (23.80%), 2 (4.76%) and 2 (4.76%) subjects from the north region, respectively ($p < 0.00$). HBV sub-genotype D2 was found to be predominant in the east region (n = 8, 32 %) followed by D3, D5 of each with 2 (8%) subjects and D1 with 1 (4 %) subject ($p < 0.00$).

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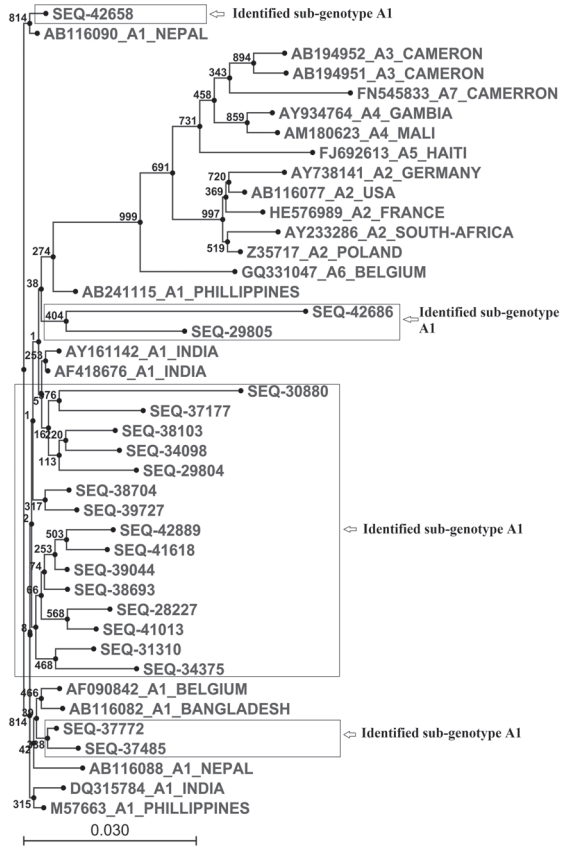


**Figure 2**: Phylogenetic analysis for the sub-genotypic distribution of HBV genotype A; The tree was formed by taking 21 HBV polymerase gene partial sequences belonging to different sub-genotypes of A1 to A7, indicated by accession numbers, followed by sub-genotypes and countries of origin. 20 subjects of HBV polymerase gene partial sequences determined in this study indicated by respective keys starting with "SEQ"

## Discussion

The most common genotype was genotype D (62.68%) that was present in these regions; supported the observation from other parts in India (3). Another genotype identified was the genotype A (29.85%) in north and east region also reported earlier in India (19 and 20), followed by genotype C (7.46%) identified only in the east region. Genotype C was dominant in neighbor countries towards the eastern part of India (21),

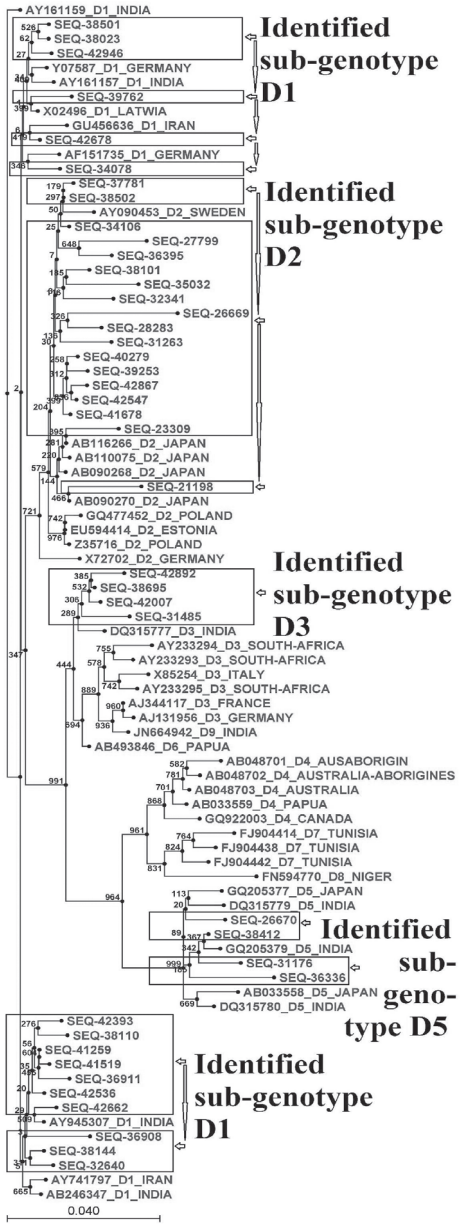


**Figure 3:** Phylogenetic analysis for the sub-genotypic distribution of HBV genotype D; The tree was formed by taking 41 HBV polymerase gene partial sequences belonging to different sub-genotypes of D1 to D9, indicated by accession numbers, followed by sub-genotypes and countries of origin. 42 subjects of HBV polymerase gene partial sequences determined in this study indicated by respective keys starting with "SEQ"

represented the distinct distribution of genotypes particularly in north and east region (22 and 23). Genotype C identified in east region in our study also, where we identified four cases of Dhaka and one case of Kolkata suggests the transmission route from eastern countries (23 and 24). HBV genotype C infection has previously been defined with more active or severe liver disease in Southeast Asia (13). Therefore, need to monitor carefully in north and east region in India as well. Genotype A was also prevalent in earlier studies (19 and 25). HBV genotypes A has less clinical significance compared to genotypes C and D, which is more prone to the development of cirrhosis and HCC (26 and 27), emphasize the major attention to stop the circulation of genotypes in these regions.

On the observation of genomic group C, we found only single sub-genotype C1. For the genomic group A, we found one sub-genotype A1, which is more prevalent in India as well as in the world (28). However, in case of genomic group D, multiple sub-genotypes D1, D2, D3 and D5 were found (Table 1) (3). In the phylogenetic tree also, the majority of the HBV sub-genotypes branched in the D1 group (38.09%, 16/42) and in the D2 group (42.85%, 18/42). However, 9.52% (4/43) of each sub-genotype branched in D3 and D5 groups (Figure 3).

To eliminate the confusion of reaction failure or to use another internal control, sequencing reactions were preceded with the same extracted materials that were used for HBV DNA viral load identification. These positive Real-time PCR results were again confirmed by targeting the HBV polymerase gene region, and recovery of amplified product on the agarose gel (Figure 1). The process has been utilized by using one set of primer, to identify and analyze genotypes as well as sub-genotypes in a single experimental run. Therefore, it is convenient and effective method to perform the experiment and analyze the results. This is done by aligning and creating phylogenetic tree of the different genotyping sequences with known sub-genotype reference sequence (Figure 2, 3, 4). In our knowledge, the above applied methodology of HBV DNA sequencing has not been demonstrated within Indian subcontinent and may be a useful target for the disease management of viral hepatitis patients

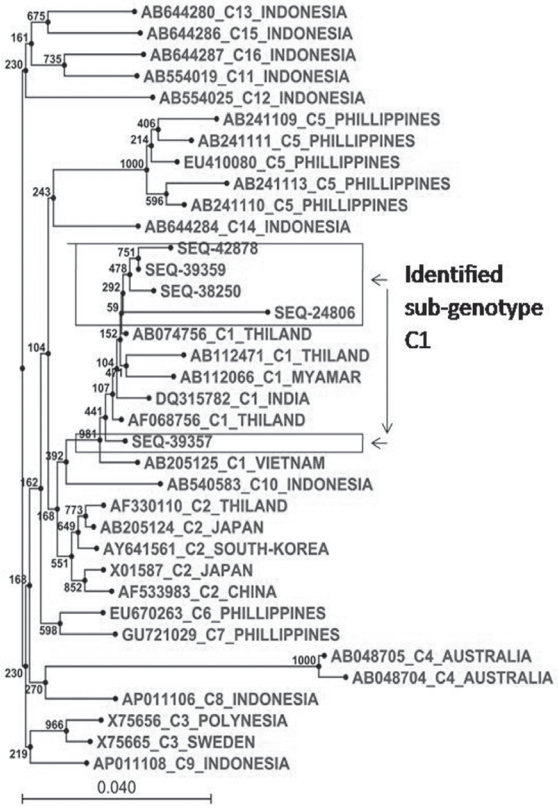


**Figure 4:** Phylogenetic analysis for the sub-genotypic distribution of HBV genotype C; The tree was formed by taking 31 HBV polymerase gene partial sequences belonging to different sub-genotypes of C1 to C16, indicated by accession numbers, followed by sub-genotypes and countries of origin. 5 subjects of HBV polymerase gene partial sequences determined in this study indicated by respective keys starting with “SEQ”

A limitation of this study was the lack of information about the previous diagnostic clinical history of the patient, whether they were carriers of HBsAg, chronic Hepatitis B or sufferings decompensate cirrhosis, and their life-style related to viral transmission.

**Table 1:** Frequency of HBV Genotypes and sub-genotypes in Indian subcontinents, East (E) and North (N)

| Region | Genotype | Sub-genotype | Counts | Frequency % | *p* Value |
|---|---|---|---|---|---|
| **E** | | | **25** | | <0.00 |
| | A | A1 | 7 | 28 | |
| | C | C1 | 5 | 20 | |
| | D | D1 | 1 | 4 | |
| | | D2 | 8 | 32 | |
| | | D3 | 2 | 8 | |
| | | D5 | 2 | 8 | |
| **N** | | | **42** | | <0.00 |
| | A | A1 | 13 | 30.95 | |
| | D | D1 | 15 | 35.71 | |
| | | D2 | 10 | 23.80 | |
| | | D3 | 2 | 4.76 | |
| | | D5 | 2 | 4.76 | |

East (E) and North (N)

## Conclusion

In conclusion, we have demonstrated the prevalence and distribution of HBV genotypes and sub-genotypes in the north and east regions of India. The method utilized of genotyping and sub-genotyping for HBV infection is useful tools to identify the epidemiology of HBV infection and to understand the clinical significance for the associations of disease progression. The high prevalence of sub-genotypes D1, D2, A1 and other sub-genotypes clearly suggest thetransmission risk to the population and circulation subjects from these regions. Also, the presence of a sub-genotype C1 in the east region and neighbor countries can be the higher transmission risk to the east region and could be the transmission route towards other regions of India. However, further analysis is required with a large number of sample volumes to define and distinguish the sub-genotypes by using complete genome sequence.

## Acknowledgments

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## Conflicts of interest

No conflict of interest.

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# Design and evaluation of terbutaline sulphate immediate release tablets prepared by fluidized bed granulation technology

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**Abstract**

**Objective:**The aim of the present study was to develop immediate release tablets of terbutaline sulphate by using fluidized bed granulation technology intended for fast action in the treatment of asthma.

**Materials and methods:**The process of granulation by fluidized bed granulation technology was used to prepare eight formulations (F1-F8) using various concentrations of binders PVP K-30, PVP K-90 and disintegrants microcrystalline cellulose, sodium starch glycolate.The granules were evaluated for preformulation parameters like bulk density, tapped density, Carr's compressibility index, and hausner ratio, angle of repose, loss on drying, and sieve analysis. The compressed tablets were evaluated for post compression parameters like thickness, hardness, weight variation, friability, disintegration, drug content, content uniformity and dissolution. The stability studies were performed for a period of 3 months at 30ºC/75%RH & 40ºC/75%RH.

**Results:** All the formulations disintegrated in less than 10 mins and released drug more than 90% in 15 mins. Formulations F4 and F8 were found to be the best formulations and was found to be stable in the varied environmental conditions.

**Conclusion:**It can be concluded that immediate release tablet of terbutaline sulphate prepared by fluidized bed granulation technique can yield a fast release tablet.

**Key words:** Terbutaline sulphate, Asthma, Fluidized bed granulator,Immediate release tablet.

## Intrduction

Asthma is a chronic disease affecting airways, results in breathlessness, tight chest, wheezing and coughing. It causes reversible obstruction of airwaysdue to constriction and inflammation (1,2,3). Terbutaline Sulphate is a FDA approved anti-asthma drug in the class of beta-adrenergics and used in acute treatment of bronchial asthma. Following an oral administration of Terbutaline sulphate, the onset of action starts within 30 minutes with a peak effect shown at 120 to 180 mins and last for 4 hrs or longer (4,5).

The Fluidized bed granulation technology is apotential one-step automated process in a closed system used in formulation development to improve therapeutic efficacy by improving the porosity and thereby dissolution of drug(6). The complete process of mixing, granulation and drying of several ingredients in a closed condition reduces the problem of material handling and shortens process time compared to other granulation processes. This enclosed process, reduces the exposure of the potent drug to the environment and complies with the cGMP (7). From

the formulation perspective, this process improves flow and compression characteristics of the powder materials, reduces segregation of varied density powder ingredients and thereby maintains content uniformity. In this technology powders are made to fluidize in a controlled air pressure inside the chamber. A binder solution or suspension is sprayed onto the fluidized particles toform agglomerates and subjected to drying at optimized conditions of temperature and air pressure.This method produces highly dispersible granules with a characteristic porous structure that enhances wettability, disintegration time and drug release of the final product. Particle size of the granules can be controlled by adjusting the quantity and droplet size of binder (8,9,10).Many articles for terbutaline sulphate first release tablets have been published by conventional granulation methods of preparation, so the present study focuses on terbutaline sulphate immediate release tablets by fluidized bed granulation technique.

**Materials**

Terbutaline Sulphate was obtained as a gift sample from AstraZeneca Pharma India Ltd, Bangalore, India. Rest of the ingredients used were of analytical grade.

**Methods**

**Preparation of Binder Solution:** A solvent mixture of isopropyl alcohol and purified water in 2:1 ratio was prepared and binder (PVP K30 / PVP K90) was added slowly to this mixture under continuous stirring at 100 to 200 rpm until a clear solution was obtained.

**Granulation and compression:**Eight different formulations of 185 mg of terbutaline immediate release tablets were prepared by varying the composition of PVP K30 / PVP K90.All the intra granular materials as mentioned in Table 1(Terbutaline sulphate, maize starch and lactosemonohydrate) were sifted through 20 mesh ASTM sieve and charged into fluidized bed processor (Pam Glatt, Germany) and dry mixed for 20 minutes. The binder solution was sprayed through spraying nozzle (top spray) as atomized liquid droplets. After complete addition of binder solution, spraying was stopped and drying was carried out till loss on drying (LOD) of granules was not more than 3.0%. LOD was checked at 60°C for 20 minutes by using infra red (IR) moisture analysing balance (Mettler Lj16, India). The critical process parameters for fluid bed granulations were maintained for all the formulationsas mentioned in the Table 2.

The dried granules were loaded in to the double cone blender (Kalweka, India). Extra granular materials like micro crystalline cellulose (MCC) or sodium starch glycolate (SSG) and Maize starch were sifted separately through 20 mesh sieveand were loaded in to the double cone Blender and mixed for 20 minutes. Magnesium Stearate was sifted through 60 mesh ASTM sieve and added to the above granules and lubricated for 4 minutes in the blender. The lubricated granules were compressed in B-tooling compression machine (Lab press, India).

**Pre compression study of the granules**

**Loss on Drying (LOD) :** Drying was carried out at set inlet temperature of 40ºC to 50ºC. Drying was continued till the product temperature reached to 45ºC. 10 g of sample was collected from sample with drawing port of FBP and LOD was recorded by using Mettler Toledo IR moisture analysing balance at 60°C for 20 minutes(11).

**Particle size analysis** : A sample of 10 g powder was placed on the top sieve. The nest of sieve was fixed to the mechanical shaker apparatus and shaken for a certain period of time (20min).The powder remaining on each sieve was weighed(12)

**Bulk Density:** It is the ratio of totalmass of powder to the bulk volume of powder and was measured by Bulk Density Apparatus, Campbell electronics, India.

**Tapped density:** The powders were tapped for 10,100 and 500 times in the bulk density apparatus, Campbell electronics, India. Tapped density is calculated as the ratio of total mass of powder to the tapped volume of powder.

**Compressibility Index :** It is the ratio of tapped density to the bulk density. It is given by

Hausner Ratio = Tapped density / Bulk density

**Evaluation of Tablet** (13,14)

**Hardness**

Hardness or diametric crushing strengthis a force required to break a tablet across the diameter. This is an indication of tablets strength to with stand the shock during handling, packaging, and shipping. The Hardness of the prepared batches of tablets were measured by Stokes- Monsanto tester.

**Friability**

Friability of the tablets was tested in Roche friabilator. 10 tablets were weighed initially and rotated at a rate of 25 rpm. After 100 rotations (4 minutes), the tablets were taken out from the friabilator and reweighed. The % friability was calculated using theformula.

F=(Winitial-Wfinal) *100/Winitial

Where F=% friability

Winitial= Initial weight of 10 tablets

Wfinal= Final weight of 10 tablets

**Thickness**

Tablet thickness was measured by verniercalipers.

**Weight Variation of tablets**

20 tablets were selected at random. The average weight was determined. The individual tablet weight was compared with the average weight of the 20 tablets.

**Table 1:** Formulation tables for terbulatine sulphate tablets

| Ingredient | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 |
|---|---|---|---|---|---|---|---|---|
| Terbutaline Sulphate (mg) | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Lactose Monohydrate (% w/w) | 61.95 | 60.95 | 60.40 | 56.65 | 61.95 | 60.95 | 60.45 | 60.95 |
| Starch maize (% w/w) | 30.00 | 30.00 | 30.00 | 33.20 | 30.00 | 30.00 | 30.00 | 30.00 |
| PVP K-30 (% w/w) | 1.00 | 2.00 | 2.50 | 2.70 | - | - | - | 1.00 |
| PVP K-90 (% w/w) | 0 | 0 | 0 | 0 | 1.00 | 2.00 | 2.50 | 1.00 |
| MCC (% w/w) | 5.00 | 5.00 | 5.00 | 5.40 | | - | 0 | 2.50 |
| SSG (% w/w) | 0 | 0 | 0 | 0 | 5.00 | 5.00 | 5.00 | 2.50 |
| Magnesium Stearate(% w/w) | 0.70 | 0.70 | 0.70 | 0.70 | 0.70 | 0.70 | 0.70 | 0.70 |
| Isopropyl Alcohol | q.s | q.s | q.s | q.s | q.s | q.s | q.s | q.s |
| Purified water | q.s | q.s | q.s | q.s | q.s | q.s | q.s | q.s |
| Total weight (mg) | 185 | 185 | 185 | 185 | 185 | 185 | 185 | 185 |

**Table 2:** Process parameters for fluid bed granulation

| Parameter | Granulation stage | Drying stage |
|---|---|---|
| Spray rate (ml/ min) | 10.00±2.00 | - |
| Atomization air (bar pressure) | 1.50±0.50 | - |
| Air blow speed (CFM) | 45.00±5.00 | 65.00±5.00 |
| Inlet temperature($^0$C) | 45.00±5.00 | 65.00±5.00 |
| Product temperature($^0$C) | 30.00±5.00 | 45.00±5.00 |
| Exhaust temperature($^0$C) | 25.00±5.00 | 55.00±5.00 |

**Uniformity of Drug Content**

Mobile Phase was prepared by dissolving 4.13 – 4.33 g of 1-Hexane sulphonic acid sodium salt in 750 ml of 50 M Ammonium formate solution and added to 250 ml of methanol followed by filtration through 0.45µm membrane filter.

Drug content was determined byion-pair chromatography on a stainless steel column of 250mm x 4.6 mm packed with octadecylsilyl silica gel bonded to porous silica 5 µ, as stationary phase in HPLC and detected at 276nm. 1-hexane sulphonic acid sodium salt in ammonium formate and methanol was used as mobile phase. Sample solution was prepared by dissolving 10 tablets in a volumetric flask with the mobile phase. Drug content was calculated and compared with the response factors for the reference standards using HPLC Agilent 1100 and 1200 series.Drug content was calculated from determining concentration of drug by the following formula

Concentration of drug in samples (µg/L) =(Vs xCs) /V

whereVs = spiked volume (ml),
Cs = spiked concentration (µg/ml),
V = Sample volume.

**Disintegration test**

The disintegration time of tablet was measured in water ($37^0$C) USP disintegration test apparatus. Three trials for each formulation were performed.

**Dissolution**

The dissolution rate was determined in simulated gastric fluid without enzymes at $37^0$C, using the USP apparatus - I rotating basket method using 900ml simulated gastric fluid without enzyme of pH 1.2 to silumate linear kinetics of absorption of terbutaline sulphate. Temperature of the dissolution medium was maintained at $37^0 \pm 0.5^0$C. Samples were withdrawn after every 5 minutes and filtered through 0.45 m filters and injected into HPLC. The percentage drug release was calculated by using HPLC Agilent 1100 and 1200 series.

The % release was calculated using the formula to find the drug content in course of time

**Stability studies**

The most satisfactory formulations of terbutaline sulphate were subjected to stability study. The stability study were carried out at two different conditions 30±2°C/75±5%RH and 40±2°C/75±5%RHfor three months. The samples were withdrawn periodically after each month and studied for physical characteristics,drug content, disintegration time and *in vitro* drug release. The data so obtained was compared with the initial data of the tablets.

**Results and Discussion**

Terbutaline Sulfate is a FDA approved anti-asthma drug. With oral administration of Terbutaline sulphate, the onset of action takes place within 30 minutes. In this study an attempt has been made to formulate aimmediate release tablet of terbutaline sulphate using fluidized bed granulation technique to promote rapid onset of action.The tablets were prepared using lactose monohydrate as diluent,starch maize as filler to keep the tablet weight 185mg constant. Microcrystalline cellulose, sodium starch glycolate were used as disintegrants, PVP K30 and PVP K 90 as binder, in a 2:1 mixture ofisopropyl alcohol and purified water as vehicle. The spray rate was optimized between 8-12 ml/min, atomization air pressure at1-2 bar, air blower speed at 40-50CFM, inlet temperature at 40-$50^0$C and exhaust temperature at 30-$40^0$C.Drying was achieved at air blower speed of 60-90 CFM, inlet temperature at 60-$80^0$Candexhaust temperatureat 60-$90^0$C.

**Evaluation of precompression parameters**

The prepared granules were studied for pre compression analysis as shown in table 3. Moisture is an important factor in compaction of blended powders into tablets. Residual moisture has impact in the flow and compression property of the granules. Tensile strength is generally low at low moisture content. Therefore,

LOD of granules (in-process) is an indicator of process end point and in the present study it was found to be low. LOD of the granules was found to be minimum within the range of 1.56-2.53%.

The preformulation study conducted on granules evaluation for flow property showed hausner's ratio below 1.14 and carr'sindex below 18.3. The hausner ratio and carr's index below 1.25 and 25 respectivelyproved the good flow properties of the powders(15). Control of particle size is essential in achieving good flow properties and proper mixing of granules and powders in tablet manufacturing. Particle size can affect a wide range of properties such as the flowability, uniformity in content, the solubility and surface area properties of a tablet formulation. The granules prepared by the fluid bed granulation technique were found to be of uniform granule size in the range of 250 to 400µm (range of sieve no 40-60) and more porous with good flow properties. The sieve analysis showed the granules were moderately coarse and the maximum retention was in sieve no 40 as shown in Figure 1.

**Evaluation of post compression parameters**

Tablet thickness was relatively constant for all the formulations. Tablet weight had some variations these variations may be attributed to the differences in bulk density in the formulations. However, all formulations were in agreement with the pharmacopoeial requirements regarding the uniformity of weight as shown in (Table 4)which showed less possibility of variations associated with the tablet press or the method of preparation (16). The hardness varied from 4.1±0.06 to 5.1±0.09 Kg/cm$^2$. Percentage Friability of all batches ranged from 0.16-0.45 % (within the limit <1%) which indicated the strength of the tablets to withstand mechanical stress during manufacturing and handling.

All the formulations were subjected to disintegration and drug release study. It revealed that all the formulations took less than 10 mins to disintegrate and released more than 90 % of drug in 15 mins as shown in table 5 and figure 2 and 3. The improvement of dissolution may be attributed to the enhancement of porosity and wettability of the granules.

Thereby thefluidised granulation process improved the flow and compression characteristics, reduce segregation, improve content uniformity and thus produced highly dispersible granules with a characteristic porous structure that enhanced wettability, disintegration time and drug release of the final product.

Comparing the disintegration and dissolution study among eight formulation, F4 andF8 with maximum dissolution profile were taken for further stability studies.

**Stability study of the optimized formulations**

F4 and F8 weretakenfor stability studies at varied conditions *i.e.,*is 30±2°C/65±5%RH. and40±2°C/75±5%RH for 3 months. No significant changes were seen in morphology, friability, drug content,

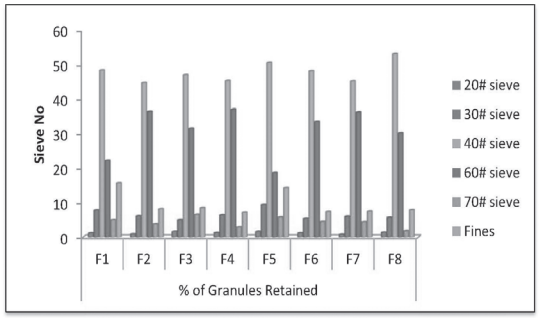


**Figure 1: Sieve analysis of precompressed granules (F1-F8)**

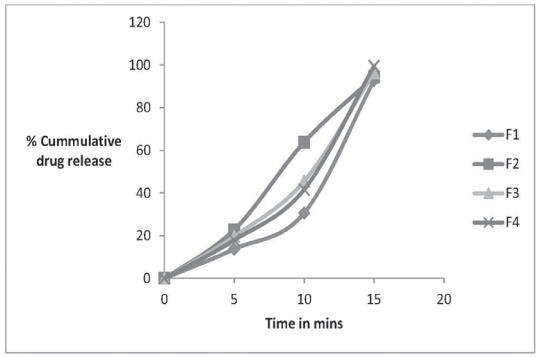


**Figure 2: Drug release studies of formulations (F1-F4)**

**Table 3:** Pre compression study

| Formula | LOD | Bulk Density g/ml | Tapped Density g/ml | Carr's index | Hausner's ratio |
|---|---|---|---|---|---|
| **F1** | 1. 56 | 0.59±0.01 | 0.67±0.01 | 12.16 | 1.13 |
| **F2** | 1.62 | 0.55±0.01 | 0.63±0.02 | 11.20 | 1.12 |
| **F3** | 1.73 | 0.55±0.01 | 0.61±0.01 | 9.80 | 1.10 |
| **F4** | 1.86 | 0.51±0.01 | 0.56±0.01 | 8.10 | 1.08 |
| **F5** | 2.20 | 0.54±0.01 | 0.65±0.01 | 16.90 | 1.20 |
| **F6** | 2.47 | 0.53±0.01 | 0.61±0.01 | 13.11 | 1.15 |
| **F7** | 2.53 | 0.50±0.01 | 0.60±0.01 | 16.60 | 1.20 |
| **F8** | 2.21 | 0.50±0.01 | 0.56±0.02 | 9.90 | 1.11 |

All values are mean ±Standard deviation (SD) and no of replicates ( n)=3.

**Table 4:** Evaluation of terbulatine sulphate tablets

| Formula | Thickness (mm) | Hardness (Kg/cm$^2$) | Friability (%) | Avg Weight (mg) | Drug Content (%) |
|---|---|---|---|---|---|
| F1 | 3.36±0.03 | 4.5±0.10 | 0.45±0.02 | 182.3±2.88 | 98.14±5.58 |
| F2 | 3.33±0.07 | 4.2±0.06 | 0.30±0.06 | 183.82±3.06 | 98.01±5.16 |
| F3 | 3.33±0.07 | 5.0±0.07 | 0.20±0.02 | 183.68±3.20 | 98.04±2.57 |
| F4 | 3.35±0.02 | 4.1±0.10 | 0.26±0.02 | 184.54±3.00 | 99.59±2.28 |
| F5 | 3.36±0.04 | 4.6±0.05 | 0.20±0.03 | 184.11±2.90 | 98.66±4.26 |
| F6 | 3.34±0.06 | 5.1±0.09 | 0.19±0.05 | 184.24±2.77 | 100.82±3.92 |
| F7 | 3.33±0.08 | 5.3±0.10 | 0.25±0.02 | 184.36±3.09 | 98.94±4.82 |
| F8 | 3.21±0.05 | 4.5±0.05 | 0.16±0.03 | 182.8±2.96 | 99.05±2.94 |

All values are mean ±Standard deviation (SD) and no of replicates ( n)=3.

**Table 5:** Disintegration and release study of terbulatine sulphate tablets

| Formula | Disintegration (Min) | % Drug release at 15mins |
|---|---|---|
| F1 | 4.30±0.05 | 92.70±0.20 |
| F2 | 3.35±0.09 | 94.40±0.50 |
| F3 | 3.30±0.12 | 96.40±0.30 |
| F4 | 3.25±0.10 | 99.40±0.30 |
| F5 | 5.05±0.08 | 95.10±0.40 |
| F6 | 7.50±0.11 | 95.60±0.30 |
| F7 | 8.10±0.10 | 90.70±0.20 |
| F8 | 4.40±0.07 | 97.00±0.40 |

All values are mean ±Standard deviation (SD) and no of replicates ( n)=3.

**Table 6:** Stability study of formulations F4 and F8 at30±2°C/75±5%RH

| Condition (30±2°C/75±5%RH) | | | | | | |
|---|---|---|---|---|---|---|
| **Formulations** | | **F4** | | | **F8** | |
| **Time Period** (month) | **1st** | **2nd** | **3rd** | **1st** | **2nd** | **3rd** |
| Hardness (kg/cm$^2$) | 4.2 | 4.2 | 4.2 | 4.5 | 4.5 | 4.7 |
| Friability% | 0.26 | 0.25 | 0.25 | 0.16 | 0.16 | 0.13 |
| Disintegration time (min) | 4.3 | 42.3 | 4.35 | 4.5 | 3.4 | 4.45 |
| % Drug Content | 99.2 | 99.4 | 99.6 | 98.2 | 98.1 | 97.4 |
| % Drug Release at 15 min | 99.5 | 99.1 | 99.4 | 97.6 | 97.1 | 96.3 |

**Table 7:** Stability study of formulations F4 and F8 at40±2°C/75±5%RH

| Condition (40±2°C/75±5%RH) | | | | | | |
|---|---|---|---|---|---|---|
| **Formulations** | | **F4** | | | **F8** | |
| **Time Period**(months) | **1st** | **2nd** | **3rd** | **1st** | **2nd** | **3rd** |
| Hardness (kg/cm$^2$) | 4.2 | 4.2 | 4.2 | 4.5 | 4.5 | 4.7 |
| Friability% | 0.26 | 0.25 | 0.25 | 0.16 | 0.16 | 0.13 |
| Disintegration time (min) | 4.3 | 42.3 | 4.35 | 4.5 | 3.4 | 4.45 |
| % Drug Content | 99.2 | 99.4 | 99.6 | 98.2 | 98.1 | 97.4 |
| % Drug Release at 15 min | 99.5 | 99.1 | 99.4 | | | |

disintegration time, and % Drug release (Table 6, and 7) at the end of the study period. Hence, it was observed that the developed Terbutaline Sulphate tablets were stable and retained their potency after stability studies.

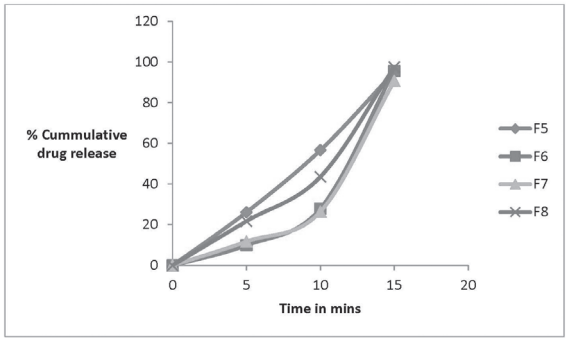


**Figure 3: Drug release studies of formulations (F5-F8)**

## Conclusion

The granules prepared by the fluid bed granulation technique were found to have uniform granule size in the range of 250 to 400µm, and a porous nature with good flow properties. The tablets prepared from these granules were compressed without any chipping, capping and sticking. Formulated tablets had given satisfactorily result for various physico-chemical evaluations of tablets like tablet dimension, thickness, hardness, friability, weight variation, and drug content. Optimized formulations F4 and F8 revealed the stability of the formulations at varied temperature and humid conditions. So it can be concluded that an immediate release tablet of Terbutaline sulphate prepared by fluidized bed granulation technique can yield a fast release tablet compare to normal granulation technology.

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# Chromatographic fingerprint analysis of piperine in polyherbal and marketed formulation by HPTLC and GC-MS methods

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**Abstract:**

The standardization of polyherbomineral formulation (PHF) is significant with regard to accessthe quality of natural medicines. The current research study highlights the chromatographic fingerprint investigation of piperine in PHF by employing GC-MS and HPTLC. PHF contain piperinewhich is utilized to take care of cough and cold. It was prepared from the mixture of Zingiber officinalis (Ginger), Piper nigrum (Kali mirch), Piper longum (Pipali), Terminalia belerica (Bahera), Terminalia chebula (Harde), Cuminumcyminum (Jira), Piper retrofractum (Chavya), Emblica officinalis (Amla), Coriander sativum (Dhaniya), sulphur, mercury, abharakbhasam and lohbhasam. The methanolic extract of both PHF and market formulation (MF) were subjected to HPTLC and GC-MS chromatographic analysis. HPTLC chromatogram finger printing of piperine in PHF demonstrated Rf values at 0.49 which was found in MF (Rf 0.47) and in standard marker (Rf 0.43). The piperine phytoconstituent present in both MF and PHF were investigated and recognized by GC-MS analysis, Thin layer chromatography (TLC), Fourier transformer infra-red (FTIR) spectroscopy and phytochemical tests. HPTLC finger printing, GC-MS analysis, FTIR and phytochemical screening tests of PHF may be useful in discriminating the species, affirm the existence of piperine phytoconstituen and act as a biochemical marker for polyherbomineral formulation. The consequence of these acquired parameters could serve as diagnostic tools to assist the regulatory authorities, scientific manufacturers and organizations for authentication and growing standard polyherbomineral formulation of high efficacy.

**Key-words:**

Piperine, Polyherbomineral formulation, Phytomarker, market formulation, Gas Chromatography-Mass Spectroscopy, High Performance Thin Layer Chromatography

## Introduction

Since traditional time, the health of human beings has been of utmost importance and market of all commodities has become global in the present era. Health pertaining marketing products have been active and prepared at distinct divisions of the globe and marketed all over the world. The necessity of standardization assures the supply of consistency of product in almost whole environment of the globe (1). WHO hooks up and aids ministry of health in endowing mechanisms for the launching of typical plant remedies into prime medical care programs, in examining efficacy and safety, and assuring sufficient resources in quality control of raw materials and manufacturing of products (2).

In order to establish the necessary framework for control of quality, safety and therapeutics effectiveness of Ayurvedic herbal formulation, there is need for standardization of manufacturing

procedures and suitable analytical techniques. Among these techniques, GC-MS and HPTLC are extensively employed to create referral fingerprints of PHF against which MF and raw substances can be analyzed and assay the final products (3,4). The finger print technique delivers the means for suitable identification, because it is specifically suited for comparison of PHF, atest based upon fingerprints in contrast to MF. From the profile of phytoconstituents, a number of phytomarkers can be selected which might be employed to further reveal the quality of the PHF. GC-MS and HPTLC have been employed for quantitative estimation of smart phytomarkers(5).

The control of quality of herbal formulation is very much tedious in contrast to synthetic drugs due to chemical complexity of herbal constituents which are responsible for pharmacological action. It is tedious to completely evaluate and identify all these compounds because Ayurvedic herbal formulations consist of hundreds of species-specific and unique substances. It is also complicated to identify accurately which usually play vital role in remedial action since these substances generally function synergistically in eliciting the therapeutic outcomes (6).

Hence, it is difficult to maintain the consistent quality from batch to batch in Ayurvedic herbal formulations because necessity and serious attention is a challenging conditional task currently. Now a days, significant initiatives have been established to control the quality of herbs along with Ayurvedic formulation through employing qualitative fingerprinting tools and/or quantitative techniques (7,8).

So, the present studies evolve to evaluate PHF and marketed formulation (MF) employing HPTLC and GC-MS techniques. PHFwas prepared from admixture of number of herbs and minerals such as Zingiber officinalis (Ginger), Piper nigrum (Kali mirch), Piper longum(Pipali), Terminalia belerica (Bahera), Terminalia chebula (Harde), Cuminum cyminum (Jira), Piper retrofractum (Chavya), Emblica officinalis (Amla), Coriander sativum (Dhaniya), sulphur, mercury, abharak bhasam and loh bhasam. HPTLC study of extract of PHF and MF were investigated to access the phytomarker and make certain relationship by contrasting their chromatogram. GC-MS analysis was also performed for investigation and identification of phytomarkers in PHF and MF.

## Materials and Methods

***Procurement of Crude Herbal Drugs*** : After checking, confirmation and authentication from Department of Botany, BabuShivnath Agrawal (BSA) PG College, Mathura, U.P., India, crude herbs were purchased from regional market, Mathura, U.P. and developed thepolyherbomineral formulation.The chemicals employed were of analytical grade in the experiment.

***Preparation of PolyherbomineralFormulation (PHF):*** PHF was produced according to the method specified in Ayurvedic Sarsangrah(9,10). Individually all ingredients were powdered and transferred through mesh (#80). Separately amount of every active powder ingredient was analyzed and blended in stipulated proportion so as to achieve uniform homogeneous mixture of PHF.

***Development of PHF and MF extracts :*** According to standard typical techniques of Ayurvedic Pharmacopoeia of India (11,12), PHF and MF (Marketed Formulation) extraction were accomplished. The extracts were produced in large quantity and gathered by using the same technique. In sterile container the extracts were preserved and stored in refrigerator till further investigation.

***Phytochemical Screening Test of Piperine :*** Phytochemical screening of PHF and MF extracts were used for the identification of piperine alkaloid. Prepare standard solution by dissolving 50 mg of extracts and piperine in 50 ml of 95% ethanol separately and shake well. The extracts and piperine were screened for various secondary metabolites by Mayer's test, Dragendroff'stest, Hager's test and Wagner's test.

**Thin Layer Chromatography (TLC) study :**

**Test Sample:** PHF, MF and piperine were added in 10ml methanol separately, and subsequently heated for 10 min, then filter and evaporated the filtrate up to 3 ml.

***Identification of piperine in PHF and MF by TLC :*** Each sample (10µl) was spotted on precoated Silica gel-G aluminium plates of uniform thickness of 0.5mm as a stationary phase. TLC was produced by employing a blend of distinct solvents; Toluene: Ethyl acetate (7:3) as a mobile phase. The development was ceasedas the solvent entrance progressed about 75 percent. UV Fluorescence light was utilized as a visualizing agent after drying the plates in air for the detection of piperine. The presence of piperine in LPF and MF formulations was diagnosed when compared in contrast to spot of standard piperine phytomarker. The spots were marked and Rf (Retardation factor) value was calculated by using following equation(11,13). The experiment was performed in triplicate for reproducibility of results.

Rf = Dsolute/Dsolvent

Where, Dsolute - Distance travelled by solute; Dsolvent- Distance travelled by solvent

***Fourier Transformer Infra-Red Spectroscopy (F.T.I.R.) Study :*** Thephytoconstituents present in a plant are specific in nature and generally do not occur in other plant. The phytoconstituent such as piperine alkaloid [Fig. 1]has specific absorption of light in infra-red region due to their different functional groups. The absorption peaks and finger print region is specific for a phytoconstituent and cannot match with another. From Infra-Red spectrum analysis, absorption peak or finger print region of PHF and MF was investigated and thepurity and presence of phytomarkerpiperine alkaloid was confirmed.

Solvent was evaporated to dryness on water bath (Accumax Equipment India Ltd., New Delhi, India) and I.R. spectrum was recorded using F.T.I.R. Spectrophotometer (Shimadzu, Japan) in the frequency range between 400-4000 cm-1 and resolution (4 cm-1) was obtainedas scanning range between wave number (cm-1) and % Transmittance.A disc of KBr (200mg) was preparedwith 2mg sample. Infrared spectrum of PHF and MF gave information about the group present in that particular compound. Therefore, I.R. spectrums of PHF and MF were compared with respect to I.R. of piperine (14) as a standard. The experiments were performed in triplicate manner to check the reproducibility.

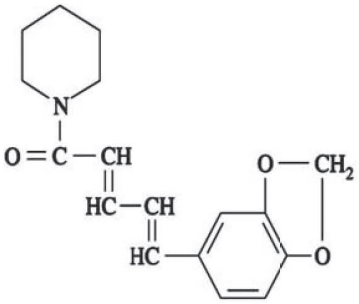



**Fig. 1.** Structure of Piperine.

**High Performance Thin Layer Chromatography (HPTLC) Study**

***Instrumentation:***

Application mode: CAMAG Linomat IV – Applicator
Filter system: Whatman filter paper (No.41)
Chromatographic conditions:
Stationary phase: Precoatedaluminium silica gel F254 plate MERCK-TLC/HPTLC
Mobile phase: Diethyl ether :Ethyl acetate : Benzene (10:30:60)
Application on Y axis
Start position: 1cm
Development on Y axis
Band length:6 mm
Chamber saturation time:30 minutes
Development mode:CAMAG TLC Twin Trough Chamber
End position:90mm from plate base
Derivatization mode: CAMAG – Dip tank for about 1minute
Visualization: 366nm, Visible, 254nm, (After spray of Anisaldehyde Sulphuric acid reagent)
Drying mode, temp. &Time:Preheated at 100 ± 5°C (TLC Plate Heater)

**Procedure**

HPTLC study of methanolic extracts of PHF and MF was performed along with the standard marker as an active constituent to ensure the presence of piperinephytoconstituent in PHF and MF. For HPTLC, each sample (2g) was extracted using methanol (25 ml) for 25 minutes on boiling water bath three times successively employing 25 ml fresh methanol and concentrated after filtration. All samples of extracts and standard were spotted on pre-coated plate (10cm×10cm with 250µm thickness) of silica gel aluminium 60F-254 employing sample applicator (CamagLinomat IV) and Hamilton syringe of 100µl. Ten millimeter from the bottom and 10 mm apart, all samples of band length (6mm) were spotted employing nitrogen aspirator at a constant application rate (15nl/s).TLC plates were dried subsequently in a current of an air dryer.The densitometric scanning was takenin the absorbance/reflectance mode on Camag TLC scanner III.

***HPTLC fingerprinting: Estimation of phytoconstituents in extracts :*** Piperine phytoconstituent was confirmed in the methanolic extract by HPTLC technique. The standard solution (1mg/ml) was prepared separately by misciblizing standard Piperine 10 mg (Sigma Aldrich, USA) in methanol (10 ml) and sample solutions of PHF and MF (1%w/v) were prepared by dissolving extracts 100 mg in 10 ml of respective solvent. Camag HPTLC system (Switzerland) was employed for analyzing the sampleswhich was equippedfor applying the samples with a sample applicator device Linomat IV, twin trough liner development chamber, Camag Scanner III attached with integration software CATS4.06 (Switzerland) and precoated aluminium silica gel F254 plate of Merck. Standard marker (Piperine) 5 µl of 1mg/ml, PHF and MF 5 µl of 10 mg/mlsolutions of extracts were placed respectively as band width (6 mm) from the edge (about 10 mm) of HPTLC plate employing applicator (CamagLinomat IV). Benzene: Ethyl acetate: Diethyl ether (60:30:10) solvent system as mobile phase was applied for investigation of Piperine. The chromatograms were developed and scanned at 254nm, 366nm and white remission using TLC scanner(15-22). For recognition and quality judgment of the formulation, HPTLC fingerprint can be utilized competently.

***Gas Chromatography-Mass Spectrometry (GC-MS) study :***

GC-MS analysis was performed employing an injector (Agilent7683 Bauto) which is coupled with a selective detector (5975 C VL Agilent mass) gas chromatography Agilent Technologies, (Santa Clara, CA) 7890A. Sample injection volume (1 µl) was employed and set at scanning rate of 2.86 scans per second. The flow rate (0.7mL/min) of carrier gas (helium grade 5) was maintained in GC and operated split less mode, 10 psi a column head pressure. On the basis of electron impact (EI) function, mass spectrometer was handled by applying 70 eV ofionization voltage and temperature 230°C. GC injector was maintained at 250°C; transmit line at 280°C. Temperature system comprised of preliminary temperature which was bring up to 250°C at a rate of 30°C/min and maintained at 70°C for 1min preceded bykeeping at 250°C for 30 min. It was maintained in 40-400 m/z scan range and attainsthe recorded mass spectra by subtraction of background and takes mean of at least five scans. The collection of retention data and chromatographic separation had been departed on a column of 30 m×0.25 mm-i.d.,which has layer of 0.25µm 100% dimethyl polysiloxane (Rtx-1) which was procured from Restek Corporation, Bellefonte, PA (23). The amount of each component was calculated in term of relative percentage by comparing its average peak area to the total areas.

***Sample preparation :*** In screw cap vials, add a weighing amount (1g) of eachPHF and MF extract separately and 10 ml methanol then kept aside for 12 hrs after sonication for 60 min.

***Identification of components :*** The identification and interpretation of the compounds was achieved by employing in-built National Institute Standard and Technology (NIST) library data bank to deal with greater than 62,000 patterns. Compare the mass spectrum of the unfamiliar test compound

against the spectrum of the reference compound recorded in the main library. The name, percentage peak area and retention time (RT) value, and structure of the components were confirmed.

**Result and Discussion**

The various specific phytochemical tests gave positive result [Table 1] for the identification of piperine in LPF and MF. Piperine alkaloid showed specific color when extracts of PHF and MF reacted with Dragendroff's, Mayer's, Wagner's and Hager's reagents.

TLC profile of PHF and MF were developed. PHF and MF showed Rf value of 4.7 and 0.48 which was near to Rf value 4.7 of piperine[Fig. 2], that indicated the presence of piperinephytomarker in PHF and MF respectively.

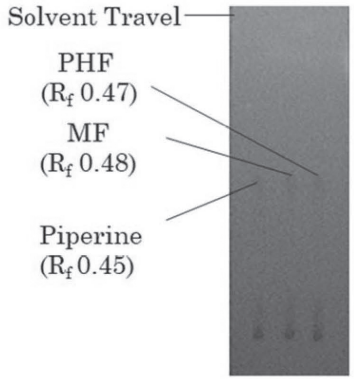



**Fig. 2:** Rf value of PHF, MF and pure piperinephytomarker.

FTIR spectrum of pure piperine exhibited various bandswhich appeared at 2865.12 cm-1 attributed to symmetric $CH_2$ stretching and at 2923.88 cm-1 asymmetric $CH_2$ stretching respectively. The band at 3070.32cm-1 indicated aromatic C-H stretching and at2923.88 cm-1 indicated aliphatic C-H stretching.Aromatic stretching of C=C and CO-N stretching showed at 1620.08 cm-1 and 1450.37cm-1 respectively. Peaks at 1027.99 cm-1 belongs to symmetric while at 1244.00 cm-1 belongs to asymmetric =C-O-C stretching and 1450.37 cm-1 showed CH2 bending and at 852.48 cm-1 showed C-O stretching. The out-of-plane phenyl C-H bending was observed at 837.05 cm-1 while 1120.16 cm-1 indicated in-plane C-H bending, results showed in [Fig. 3] (14). The piperine phytoconstituent was found to be present in both PHF and MF thus it reveals good relationship between them.

HPTLC analysis of PHF and MF extracts were performed to assure the presence of piperineas well as relationship between them. In Fig. 4 HPTLC fingerprint of extracts of PHF, MF formulation and standard (piperine) are depicted. Rf values 0.49, 0.47 and 0.43 were detected in chromatogram of extracts of PHF, MF and standard piperine respectively, as showed in [Fig 4-5]. It was noticed that the chromatogram of the PHF coordinated accurately with that of the MF. Thus, HPTLC study confirmed good correlation between both PHF and MF extracts and confirmed the presence ofpiperine (24).

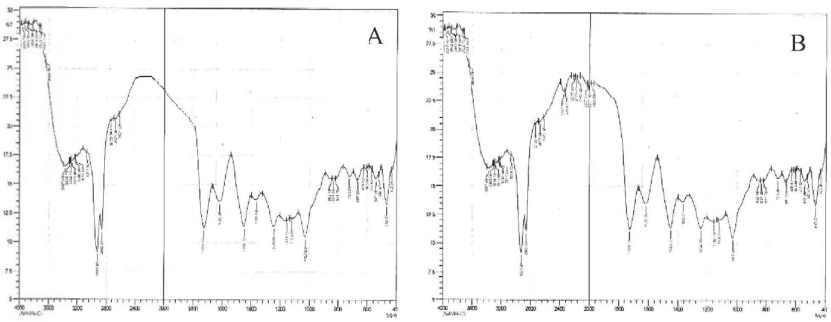
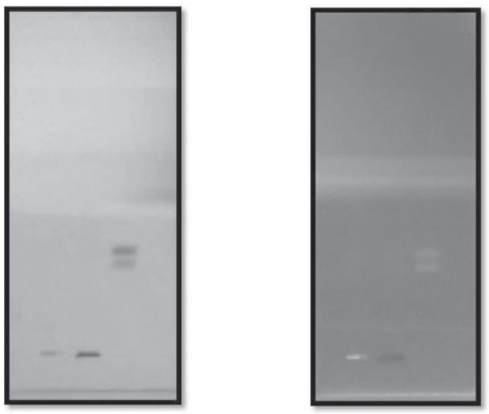



**Fig.3.** FTIR spectrum of (A) PHF; (B) MF.

Chromatographic fingerprint analysis of piperine in by HPTLC and GC-MS



(A) (B)

**Fig. 4.** HPTLC chromatogram of (A) at 254 nm contains PHF, MF and standard piperine and (B) at 366 nm containsPHF, MF and Standard Piperine.

The presence of phytoconstituent in the PHF and MF was also recognized by GC-MS method. From [Fig. 6-7], the chromatogram of PHF and MF showed the presence of several peaks but piperine peak (10.88% peak area) in PHF extract was found to be at 20.668 retention time and in MF extract, piperine peak (2.38% peak area) was found to be at 20.609 retention time. The substances relating to peaks were investigated by accessing data of the NIST library of peaks and mass spectra of the peaks with those reported in literature. The piperine phytoconstituent was found to be present in both PHF and MF extract thus proving good relationship between them (25).

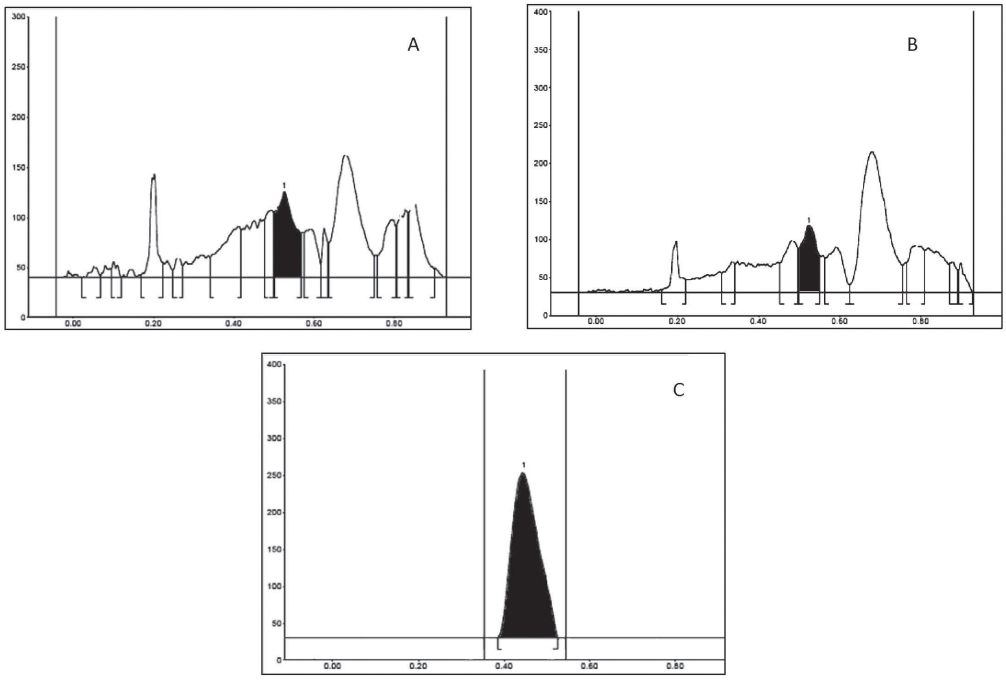



**Fig. 5.**HPTLC chromatogram of (A) PHF, (B) MF and (C) Standard Piperine.

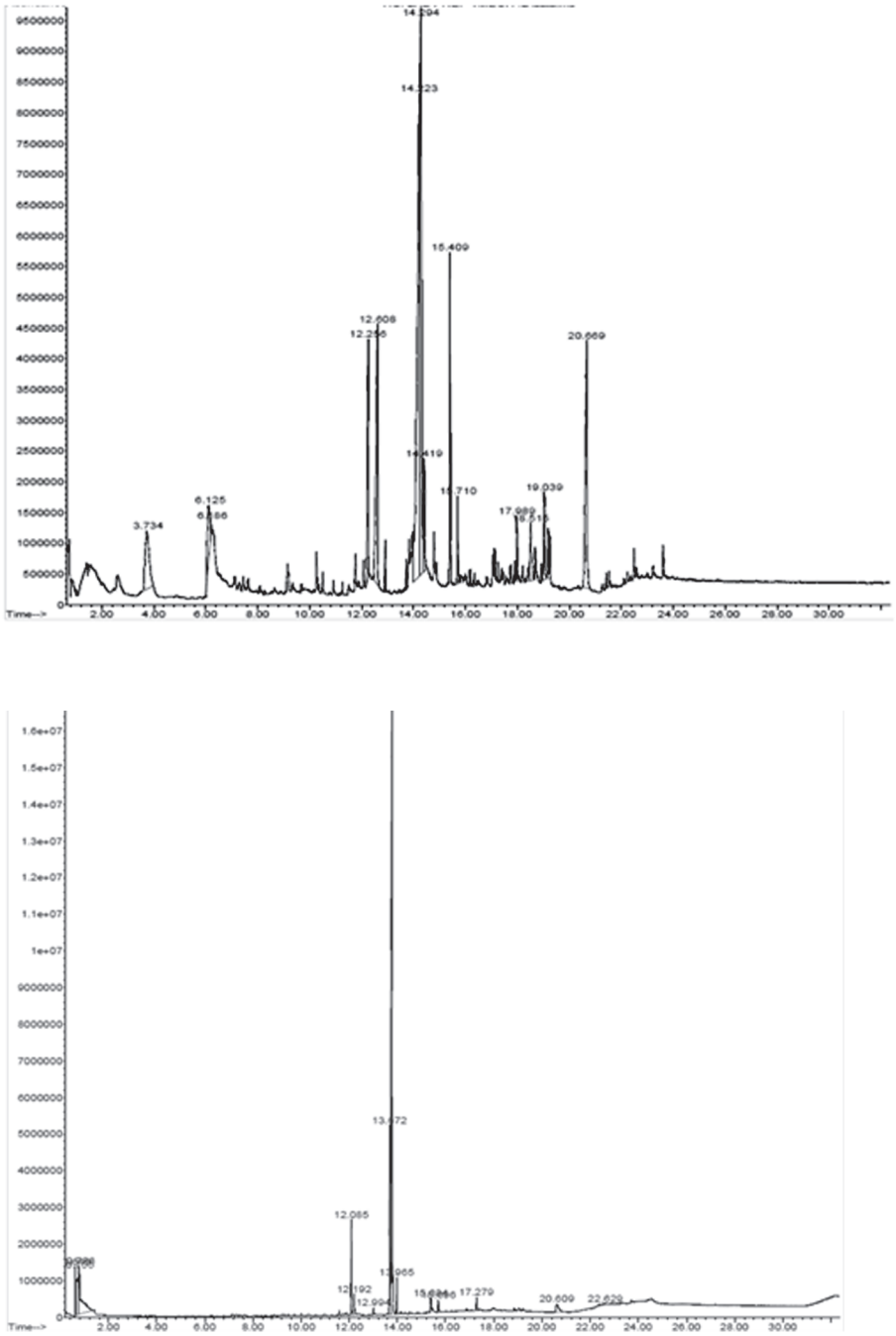



**Fig. 6**. Gas Chromatography-Mass Spectroscopyspectrum of (A) PHFand (B) MF.

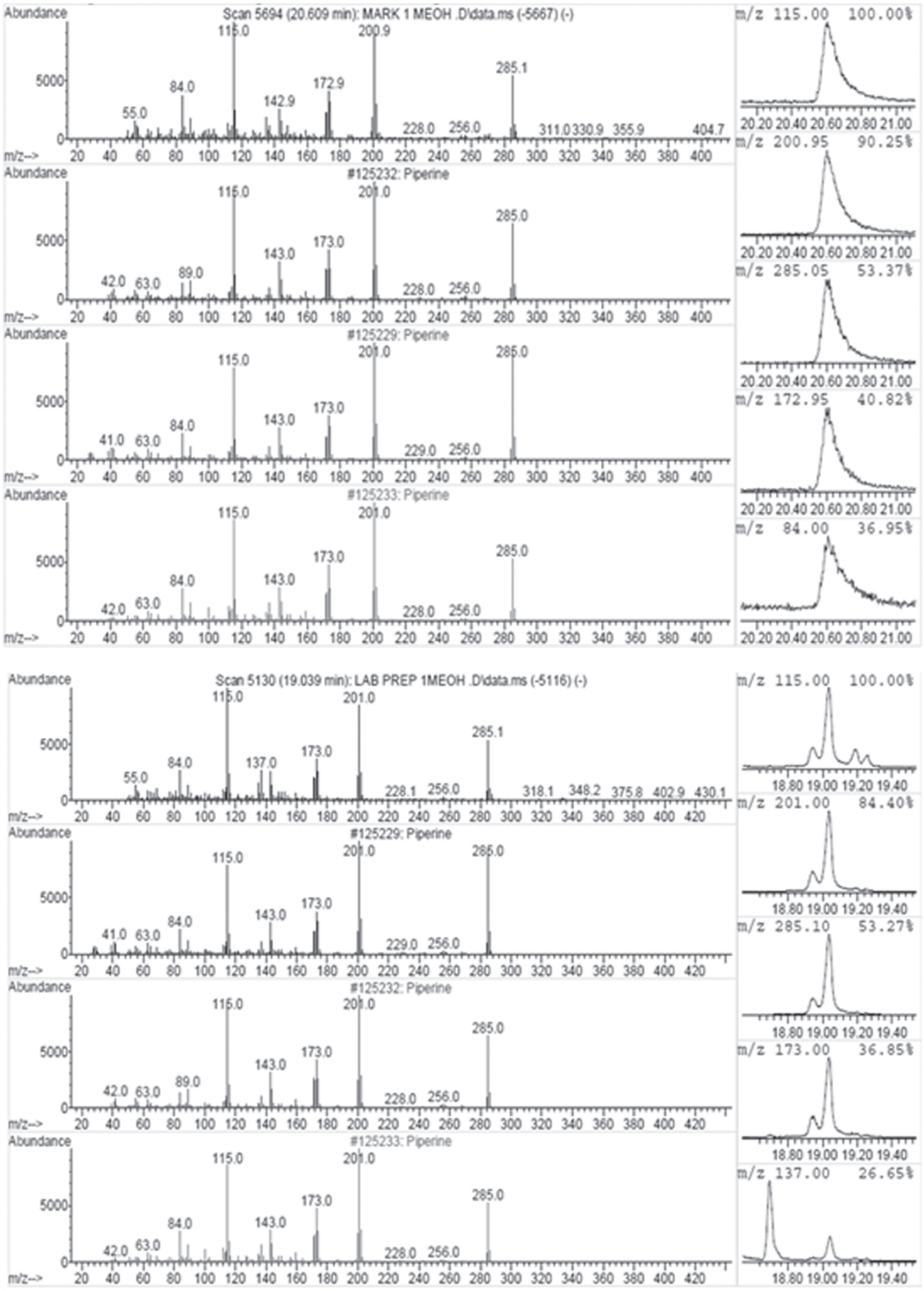



**Fig. 7.** Mass spectrum of (A) MF and (B) PHF.

**Table 1.** Various specific Phytochemical tests for identification of piperine in PHF and MF.

| Test/Reagent | Colour | MF | PHF | Piperine |
|---|---|---|---|---|
| Wagner's reagent | Reddish brown precipitate | + | + | + |
| Hager's reagent | Yellow precipitate | + | + | + |
| Mayer's reagent | Dull white precipitate | + | + | + |
| Dragendroff's reagent | Orange red precipitate | + | + | + |

'+': Presence, '-': Absence, MF: Marketed Formulation, PHF: Polyherbomineral Formulation

## Conclusions

Phytochemical screening, TLC, FTIR, HPTLC finger printing and GC-MS analysis of poly herbomineral formulation (PHF) may be useful in discriminating the species, affirm the existence of phytoconstituents such as piperine whichact as a phytomarker for polyherbomineral formulation. The consequence of assessment of these tests could serve as analyzing tool to assist thescientific manufacturers, regulatory bodies, and organizations for authentication and growing standard traditional polyherbomineral formulation (PHF) of outstanding quality and therapeutic efficacy.

## Conflict of interest

The author has no conflict of interest on this article.

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# A comparitive study on levels of sirt 1 and antioxidant status in type 2 diabetic and diabetic nephropathic patients - A case control study

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**Abstract**

Diabetic nephropathy is a leading cause of end-stage renal failure worldwide. Its morphologicc haracteristics include glomerular hypertrophy, basement membrane thickening, mesangial expansion, tubular atrophy, interstitial fibrosis and arteriolar thickening. All of these are part and parcel of micro vascular complications of diabetes. Previous study evidences indicates that oxidative stress is the common denominator link for the major pathways involved in the development and progression of diabetic micro- as well as macro vascular complications of diabetes. SIRT1 deacetylates target proteins using the coenzyme $NAD^+$ and is therefore linked to cellular energy metabolism and the redox state through multiple signalling and survival pathways. SIRT1 deficiency under various stress conditions, such as metabolic or oxidative stress or hypoxia, is implicated in the pathophysiologies of age-related diseases including diabetes, cardio vascular diseases, neurodegenerative disorders and renal diseases.

**Objective**

The present study is one such attempt to find the relation between SIRT 1 levels and antioxidant status in diabetic nephropathy in ethnic south Indian population. In the present study, we focus on the protective functions of sirtuins and the association of sirtuins with the pathophysiology of renal diseases, including diabetic nephropathy.

**Methodology :** In the present study, 30 cases presenting with diabetic Nephropathy and 30 ageand sex matched controls with Type 2 diabetes were included in the study.

**Results :** We found there was significant increase in the levels of all parameters such as MDA, SOD, GPx, GR, and SIRT1 in Diabetic Nephropathy patients when compared with Type 2 diabetes Mellitus subjects.

**Conclusion :** This study revealed that Sirt 1 plays a role in susceptibility to diabetic nephropathy patients with type 2 DM.Therefore the activation of SIRT1 in the kidney may be a new therapeutic target to increase resistance to many causal factors in the development of renal diseases, including diabetic nephropathy.

**Keywords:** Diabetes, Nephropathy, SIRT 1, Super oxide dismutase, Glutathione peroxidase,

**Introduction**

Diabetes mellitus (DM) is a major medical problem worldwide. It is the underlying cause of micro vascular disorders such as diabetic nephropathy and retinopathy and macro vascular diseases such as coronary artery and peripheral

vascular diseases. Currently, more than 347 million people worldwide are suffering from DM (1). The increased prevalence of DM has led to a significant increase in the prevalence of diabetic kidney disease (DKD) with estimates that 44% of all new end stage renal disease (ESRD) cases in US are due to DKD (2, 3). Several factors including hyperglycemia, insulin resistance, renal lipid accumulation, inflammation, and activation of the renin– angiotensin system (RAS) are involved in the pathogenesis of DKD (4) and they activate multiple signaling pathways resulting in kidney cell injury and the development and progression of the disease (5, 6). Since the discovery of the silent information regulator 2 (Sir2) family and its beneficial effects on aging (7, 8), scientists have shown that the homologs of Sir2 in higher eukaryotic organisms, known as Sirtuins (SIRTs), are a conserved family of a nicotinamide adenine dinucleotide (NADC)-dependent deacetylases/ mono-ADP ribosyltransferases that are associated with numerous cellular signaling pathways that include senescence (9–12), apoptosis (13), DNA damage repair (14), and autophagy (12, 15). By far, SIRT1 is the most studied member of this family and its protective roles against kidney injury are well established, making it a promising candidate for targeted therapies to halt disease progression.

Diabetic nephropathy is a serious micro vascular complication of diabetes, and is a leading cause of end-stage renal disease in Western countries (16). The escalating prevalence and limitation of currently available therapeutic options highlight the need for a more accurate understanding of the pathogenesis of diabetic nephropathy. According to world health organization it is the seventh leading cause of death by 2030 (17). The prevalence of diabetic nephropathy was higher in Asians, Africans and Americans. In India, the prevalence of diabetic nephropathy is 2.2% (5). As per International Diabetes Federation (IDF), total number of people with diabetes are about 69.2 million and it may raise to 123.5million by 2040 (18).

**Role of SIRT 1 in diabetic nephropathy**

Sirtuin is a nicotinamide adenine dinucleotide–dependentdeacetylase. One of its isoforms, Sirt1, is a key molecule inglucose, lipid, and energy metabolism. The renal protective effects of Sirt1 are found in various models of renal disorderswith metabolic impairment, such as diabetic nephropathy. Protective effects include the maintenance of glomerularbarrier function, anti–fibrosis effects, anti–oxidative stresseffects, and regulation of mitochondria function and energymetabolism. Various target molecules subject to directdeacetylation or epigenetic gene regulation have beenidentified as effectors of the renal protective function ofsirtuin. Recently, it was demonstrated that Sirt1 expressiondecreases in proximal tubules before albuminuria in a mousemodel of diabetic nephropathy, and that albuminuria issuppressed in proximal tubule–specific mice over expressingSirt1. These findings suggest that decreased Sirt1 expression in proximal tubular cells causes abnormal nicotine metabolism and reduces the supply of nicotinamide mononucleotide from renal tubules to glomeruli. This further decreases expression of Sirt1 in glomerular podocytes and increases expression of a tight junction protein, claudin-1, which results in albuminuria. Activators of the sirtuin family of proteins, including resveratrol, may be important in the development of new therapeutic strategies for treating metabolic kidney diseases, including diabetic nephropathy.

Oxidative Stressplays a major role in pathogenesis of diabetic nephropathy. It is caused by an imbalance between a relative overload of oxidants and a depletion of antioxidants (12). Sirtuin 1 expression is decreased in conditions like chronic metabolic stress, oxidative stress or hypoxia that drives the pathophysiologies of age related diseases which includes CVS, diabetes and renal diseases. As the disease progresses, antioxidant potential decreases, and the plasma lipid peroxidation products increase depending upon the level of glycemic control. Increased oxidative stress has been associated with aging, and SIRT1 has been shown to combat oxidative

stress by modulating transcriptional activities of several key proteins involved in oxidative stress response and mitochondrial biogenesis.

The aim of the present study is to find the relation between SIRT 1 level and antioxidant status in diabetic nephropathy in South Indian population.

## Materials and Methods

### Study design and Ethical clearance

The present study was conducted in the Department of Biochemistry in collaboration with Department of Nephrology in Saveetha medical College, Thandalam, Chennai. The study was conducted on patients with Type 2 Diabetes mellitus and Diabetic Nephropathy admitted in the nephrology unit in Saveetha Hospital and Medical College. This study was approved by Institutional Human ethics Committee.

### Type of study

It is a Case –Control Study.

### Sample size

Study population consisted of 30 patients with Diabetic nephropathy (Age range 40-75 yrs ) and control group consisted of 30 patients with Type 2 Diabetes Mellitus who are on medical treatment without any complications.

### Inclusion criteria

· Cases : Known diagnosed patients of Diabetic Nephropathy attending the department of nephrology of saveetha medical college. (defined as patients having arterial hypertension less than 200/160,eGFR > 45 and <90 mL/min/1.73 m2 and/or urinary albumin:creatinine ratio >3 mg/mmol(15)

· Controls: Known diabetes mellitus patients who are on medical treatment without any complications as controls

· Age Group of 40-70yrs for both cases & controls

### Exclusion criteria

Patients will be excluded if they have any of the following:

- a history of cardiovascular disease, defined as having a clinical record of ischemic heart disease (angina, myocardial infarction, coronary artery revascularization and or heart failure),
- peripheral vascular disease (intermittent claudication or peripheral artery revascularization) or
- cerebrovascular disease (transient ischemic episodes or stroke),
- a history of malignancy or any other life threatening illness, current pregnancy,
- systolic blood pressure >200 mmHg,
- diastolic blood pressure >160 mmHg, hemoglobin A1c > 10 %,
- Significant renal impairment (eGFR< 45 mL/ min 1.73 m2) and nephrotic range urine protein excretion (total protein excretion rate >3 g/day or albumin:creatinine ratio >300 mg/ mmol).
- Patients with age <40 and >70 are excluded (15).

**Sample collection and storage :** 5ml of venous whole Blood and EDTA samples were collected from both Type 2 Diabetes Mellitus and Diabetic Nephropathy.

**Biochemical analysis:** Malondialdehyde (MDA), Superoxise Dismutase (SOD), Glutathione Peroxidase (GPx), Glutathione Reductase (GR) and Sirtuin 1 (Sirt1) levels were estimated by ELISA (Enzyme linked Immunosorbent Assay) using Robonik ELISA reader instrument.

**Stastical analysis :** Statistical analysis were done using student t – test and p-value significance. P-value <0.01 were considered as significant.

## Results

In the present study a total number of 60 subjects comprising of 30 Type 2 Diabetes Mellitus patients ((Control) Group-I ) and 30 Diabetic Nephropathy cases (group-II) were included.

In the present study, we identified that association between SIRT1 and oxidative stress

is nominally associated with susceptibility to diabetic nephropathy.

In the present study, there was significant increase in the Microalbuminuria excretion ratio in the Diabetic nephropathy patients when compared with diabetic patients. (Table 1).

We found that, the levels of lipid peroxidation product Malondialdehyde were significantly high in diabetic nephropathy cases (8.06) when compared with diabetes mellitus (3.71) patients.

In the present study, the levels of antioxidant enzymes statistically significantly decreased in diabetic nephropathy patients (SOD-58 ± 11; GPx-45 ± 18.2; GR-10 ± 2.54) when compared with normal diabetic cases (SOD-60±13.5; GPx-49±13.4; GR-12± 2.60).

In the present study, we observed SIRTUIN 1 levels were also significantly decreased in diabetic neprhopathic patients When compared with diabetic patients.(D-3.0±0.7; DN-2.0±0.66).

**Discussion**

Diabetic nephropathy is characterized by albuminuria (>300mg/day) and a reduced GFR (19).

The present findings revealed that there was significant increase in albumin levels in DN cases when compared with type 2 diabetic patients (Table 1).

It should be considered that the albuminuria is sometimes present at the moment when DM is diagnosed, after the kidney has been exposed to chronic hyperglycemia since the prediabeticphase.Themechanisms implicated in the pathogenesis ofDN are multiple and complex. The first hemodynamic changes of glomerular hypo perfusion and hyper filtrationfavour theleakage of albumin from the glomerular capillaries.

Oxidative stress results from the link with the majority of molecular events that underline the pathological process in DN. It is related to alterations in the redox state caused by the persistent hyperglycemic state and the increase in AGEs. These events affect the renin-angiotensin system and the signalling of the transforming growth factor-beta (TGF- 5ØýÞ), producing chronic inflammation and glomerular and tubular hypertrophy. The renal fibrosis is due primarily to the

**Table1.** Comparison of Microalbuminuria ratio in Diabetic nephropathy cases and Type 2 diabetic patients (control).

| Clinical Parameter | Diabetic Nephropathy patients (Mean±SD | Type 2 diabetic patients (Mean±SD) |
|---|---|---|
| Microalbuminuria | 306.55±20.54 | 24.75±4.00 |

**Table 2.** Comparison of antioxidant enzyme status and SIRT1 levels in Type 2 Diabetic mellitus (Control) and Diabetic nephropathy patients (Cases).

| S.NO | Antioxidant Enzymes | GROUP-I = 30 Mean ± SD n | GROUP-II n = 30 Mean ± SD | p-Value |
|---|---|---|---|---|
| 1 | MDA | 3.71± 2.3 | 8.06 ± 6.19 | 0.0006 |
| 2 | SOD | 60±13.5 | 58 ± 11 | 0.5318 |
| 3 | GPx | 49±13.4 | 45 ± 18.2 | 0.3364 |
| 4 | GR | 12± 2.60 | 10 ± 2.54 | 0.0038 |
| 5 | SIRT 1 | 3.0± 0.7 | 2.0± 0.66 | < 0.0001 |

accumulation of the mesangial cells, favouring the depositing of extracellular matrix (ECM), the thickening of the tubular and glomerular membranes, the dysfunction of podocytes, and the appearance of apoptosis.

Oxidative stress in DN has the ability to act as a trigger, modulator, and link within the complex web of pathological events that occur in DN. There are various molecular events that underlie and connect the metabolism, inflammation, and the oxidation in DN.It is demonstrated that the main cause of morbidity and mortality in patients with CKD is due to CVD and that the oxidative stress together with the subclinical inflammatory state is ultimately responsible for the generation of atherosclerotic plaque (20).

In the present study revealed that there was significant decreased levels were observed in diabetic nephropathy cases.

The various body organs, particularly the kidney, suffer from different degrees of age-related damage. The kidney is vulnerable to specific age-related injuries. Therefore, the incidence of chronic kidney diseases develops along with age. Aging often leads to increased oxidative stress, free radical generation, and decreased antioxidant and free radical-scavenging activities.

These findings suggest that oxidative stress is a significant cause of chronic kidney diseases. In the present study there were significant increased levels of MDA in DN cases comparedwith Type 2 diabetic cases (Table 2).

MDA has been documented as a primary biomarker of free radical mediated lipid damage and oxidative stress (21). Significant changes in lipid metabolism and structure have been reported in diabetes, particularly in patients with vascular complications (22). Increased level ofMDA in diabetics suggests that peroxidative injury may be involved in the development of diabetic complications. The increase in lipid peroxidation is also an indication of decline in defence mechanisms of enzymatic and non-enzymatic antioxidants (23).

MDA increase confirms that it is associated with increased production of reactive oxygen species and free radicals. Our study correlated with previous findings. (1, 13, 14).

Superoxide Dismutase, a superoxide scavenging enzyme which is considered the first line of defence against deleterious effect of oxygen radical in the cells. Which is decreased in diabetic nephropathy when compared to type 2 diabetes and it is not statistically significant (Table 2).

A selenium containing enzyme, Glutathione Peroxidase is also decreased in diabetic nephropathy when compared to type 2 diabetes and it is not statistically significant. GR levels in diabetic nephropathy are decreased when compared with T2D and it is statistically significant (Table 2).

SIRT1expression changes under different physiological and morbid conditions. It is decreased in conditions of chronic metabolic stress, oxidative stress, or hypoxia that drives the pathophysiologies of age related diseases including diabetes, cardiovascular, and renal diseases. In aging kidneys both the expression and activity of SIRT1is decreased due to age associated reduction in systemic NADC biosynthesis (12). Similarly, reduction in SIRT1expression was observed in kidney glomeruli and tubule interstitial compartments of patients with mild to severe DKD, which was inversely correlated with the histopathological severity of the renal disease and with the amount of proteinuria (24, 25).

SIRT1 is a member of NAD+ -dependent histone deacetylase, which involves in various nuclear events such as transcription, DNA replication, and DNA repair. SIRT1 plays an important role not only in the regulation of aging and longevity, but also in the development and/or progression of age-associated metabolic diseases, such as type 2 diabetes. The effects of SIRT1 polymorphisms on susceptibility to diabetic nephropathy might be mediated by differences in the metabolic state among individuals, including glycemic control, obesity, blood pressure.

As sirtuin 1 is involved in several energy homeostasis pathways it is considered as master regulator. Prior studies showed the associations between *SIRT1* and oxidative stress.

SIRT1 can protect cells from apoptosis induced by oxidative stress. Hao and Haase (26) observed that SIRT1 is over expressed when renal medullary interstitial cells are exposed to high-permeability and low oxygen environments. Down regulated SIRT1 expression significantly reduces oxidative stress resistance and triggers massive apoptosis. Conversely, activated SIRT1 promotes cell survival. This finding was verified in an in vitro unilateral urethral obstruction model. SIRT1 directly or indirectly controls the activation of FOXO1, FOXO3, and FOXO4 through deacetylation and regulates cell response to oxidative stress (27).

Oxidative stress is hypothesized to play a role in the development of diabetes with and without nephropathy. Oxidative stress has been considered to be a pathogenic factor of diabetic complications including nephropathy.

High intracellular glucose concentration has been suggested to be a prerequisite for the development of functional and structural changes in the kidney typical of diabetic nephropathy. Under the conditions of intracellular hyperglycemia, the cellular NADH/$NAD^+$ ratio is decreased.

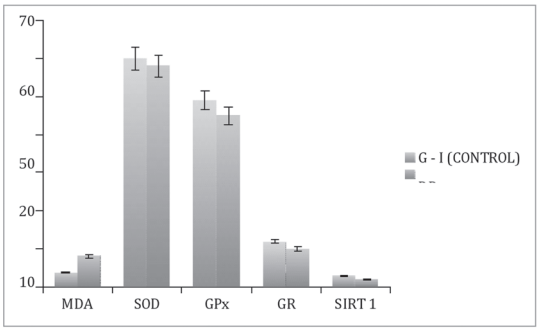


**Figure 1:** Biochemical Variations in cases (Diabetic Nephropathy) and Controls (Type 2 diabetic patients).

Antioxidant therapy is one of the most important treatment strategies for diabetic patients without nephropathy for the prevention and slowing of diabetic nephropathy before reaching to End Stage Renal Disease.

Vascular endothelial growth factor (VEGF) is a protein secreted by the podocytes and the mesangial renal cells under oxidative stress situation which plays a role in the extension of diabetic kidney disease.

Reactive oxygen species are reduced by Sirtuin, by modulating the acetylation of respiratory chain and by stimulating mitochondria superoxide dismutase and isocitrate dehydrogenase which generates NADPH for glutathione pathway.

The decreased levels of antioxidants which in turn decreases the level of SIRT 1 in diabetic nephropathy when compared to Type 2 Diabetes. It is statistically significant (Table 2).

## Conclusion

In recent decades, numerous investigators have made efforts to identify the molecular mechanisms involved in the initiation and progression of diabetic nephropathy to develop new therapeutic strategies. However, end-stage renal failure due to diabetic nephropathy continues to increase worldwide. There is an urgent need to identify new therapeutic targets to prevent diabetic nephropathy. The present findings revealed that there was significant decrease in antioxidant enzymes and SIRT 1 in Diabetic nephropathy patients. These studies revealed that further investigation into the targets and functions of other sirtuins will help develop new strategies for protection against renal diseases.

## Acknowledgement

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**Conflict of Interest**

The authors declare that there is no conflict of interest in this study.

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# Plackett-Burman design for screening of fermentation process parameters and their effects on L-methionine production

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**Abstract**

In the present study, economical fermentation medium process was developed to achieve the maximum production of L-methionine by *Corynebacterium glutamicum* through screening of different nutritional and physical parameters by Plackett-Burman design. A total of eleven process variables such as plantain as carbon source, groundnut as nitrogen source, $CaCO_3$, $K_2HPO_4$, $KH_2PO_4$, biotin, $MgSO_4.7H_2O$, inoculum size, agitation speed, volume ratio of medium/fermenter and pH were used for screening experiments. The PBD model results suggested that seven variables namely plantain as carbon source, groundnut as nitrogen source, $CaCO_3$, $MgSO_4.7H_2O$ and $KH_2PO_4$ had shown a significant effect on L-methionine production, while remaining six variables didn't show a much effect on L-methionine production. The $R^2$ value (0.99) of analysis of variance (ANOVA) recommended that the model used for response prediction is significant ($p<0.05$). In comparison with the unoptimized medium, 24% higher L-methionine production was obtained from the optimized medium and L-methionine production was found to be 5.6 g/l.

**Keywords:** L-methionine, *Corynebacterium glutamicum*, Optimization, Plackett-Burman Design.

## Introduction

Methionine, alpha-L-amino-gamma-methylthio-nbutyric acid is nutritionally essential for mammals and fowls. It can't be synthesized internally, but may be added to food and feed materials to improve the protein quality (1). Methionine is generally being produced by chemical and enzymatic methods, both are expensive, chemical method requires hazardous chemicals and enzymatic method requires expensive enzymes. Methionine can be produced economically by using fermentation, because many fermentation processes have been developed to produce many other amino acids inexpensively (1, 2 and 3).

Plant proteins are frequently deficient in methionine and consequently an exclusively vegetable diet may fail to meet nutritional requirements. Methionine deficiency has been linked to development of various diseases and physiological conditions including toxemia, childhood rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia, Parkinson's liver deterioration, and impaired growth (4). Deficiencies can be overcome by supplementing the diet with methionine and, therefore, methionine is of significant interest (5).

The history of species *Corynebacterium* as amino acid producer started in the 1950s when

Dr. Kinoshita was the first to discover that *Corynebacteria glutamicum* is a superior amino acid producer (6,7 and 8). Now a day's L-glutamic acid, Llysine, L-isoleucine, L-threonine, L-aspartic acid and Lalanine are produced by *Corynebacteria* in terms of high production rate and economical value.

In this work, we carried out the screening of critical medium fermentation components and conditions, which have been predicted to play a significant role on methionine production by *Corynebacteria glutamicum* using Plackett-Burman design. Design expert 8.0.7.1 (Stat-Ease) statistical software was used to carry out Plackett-Burman design, statistical analysis of results and coefficient of the effect estimate.

## Materials and Methods

### Chemicals

All the chemicals and reagents were purchased from Hi-Media, Mumbai. Plantain and groundnut were obtained from the local market in Guntur, Andhra Pradesh, India.

### Microorganism and Culture conditions

L-Methionine producing strain of *C.glutamicum* MTCC 2745 obtained from the microbial type collection centre, Chandigarh, India was used throughout this study. It was maintained on nutrient agar slants (Beef extract 1 g/l; Yeast extract, 2 g/l; Peptone, 5 g/l; Nacl, 5 g/l; Agar, 15 g/l and pH was adjusted to 7.2 with 1N NaOH) and stored at refrigeration temperature 4°C for further analysis. 3 ml of 24 hour slant culture was used to inoculate a 100 ml Erlenmeyer flask containing 30 ml of seed medium.

### Design of Experiments

The nutrient and physical parameters such as plantain as carbonsource, groundnut as nitrogen source, $CaCO_3$, $K_2HPO_4$, $KH_2PO_4$, biotin, $MgSO_4.7H_2O$, inoculum size, agitation speed, volume ratio of medium/fermenter and pH were used for experimental screening purpose. Fermentation (shake flask) experiments were designed as per the PBD matrix (Shown in Table.2) based on 30 mL of medium dispensed into 100 mL Erlenmeyer flask. Fermentation mediumwas sterilized at $121^0$C and 15 min. Upon cooling, the inoculum was added to the media and flasks were incubated in an orbital shaker at 170 rpm. All measurements were done in triplicates and average values were reported. The effect of individual components on L-Methionine production was calculated by following equation.

$$E = \frac{2\left(\sum H^{+} - \sum H^{-}\right)}{N}$$

Where E is the effect of parameters and $H^+$ and $H^-$ are responses of trails in which the parameter high and low levels respectively and N is the number of trails.

### Analytical Techniques

### Carbon sources (Preparation of starches)

Agriculture products utilized here for the preparation of starch is plantain. Starch was prepared according to the method portrayed by (9). Plantain samples were brought from Guntur (Andhra Pradesh, India) local market were first peeled, washed and cut into little pieces before being homogenized with water in Moulinex blender. Homogenate blended with excess water was tied in cheese cloth and placed on tripod stand overnight, to take into account extraction of starch into a clean plastic bowl. The supernatant was emptied and the sedimented starch dried at 50°C for 48 hours. The resultant chips were grounded into powder and utilized as starches.

### Saccharification of starch

Saccharification of starch took after the method illustrated by (2). A 500 ml flask containing a mixture of 30g of starch and 100 ml of water was heated for 15 min at 95°C in a water bath to gelatinize starch. The beaker was covered with aluminium foil after adding 1 ml of á-amylase and again heated in water bath for 10 min at 95°C to impact liquefaction. After cooling liquefied starch to 60 °C, 1 ml amyloglucosidase enzyme was added before replacing the beaker in the water bath at 60 °C for 48 hr for saccharification to takes place.

**Nitrogen sources: Preparation of defatted proteins**

The protein utilized here from agricultural products as nitrogen sources is groundnut. For preparation of defatted protein took after the strategy explained by study (2). Groundnut was crushed in a blender and then some division of homogenized proteins was defatted by soxhlet extraction method using diethyl ether. The meals obtained after extraction were oven dried at 34-35$^{o}$C for 20 hr and afterward ground into fine powder.

**L-Methionine Assay**

Quantitative determination of L-methionine in the culture broth without purification was carried out by the modified calorimetric method (10). A 5 ml volume of the culture broth was centrifuged at 5,000xg for 20 minutes and the cell free supernatant was assayed for Lmethionine.

1 ml of 5N NaOH was added to a test tube followed by the addition of 0.1ml of 10% sodium nitroprusside solution with thorough mixing. The mixture was allowed to stand for 10 min. Then two milliliters of 3% aqueous solution of glycine was added to the reaction mixture with frequent shaking over a period of 10 min. After an additional 10 min interval, 2ml of concentrated *ortho*-phosphoric acid was added drop wise to the mixture with shaking. Colour development was allowed to proceed for 5 min and the colour intensity measured at 540nm in a spectrometer. A blank containing distilled water and all other reagent served as the 100% transference standard. Results obtained with the test samples were interpolated on a standard methionine curve.

**Estimation of Reducing Sugar**

The reducing sugar (glucose) in the time-course fermentation broth was estimated by the modified method described by (11). A 1ml volume of dinitrosalicylic acid was added to 1ml of the supernatant in a test tube and the mixture heated in boiling water for 10 minutes. The test tube was cooled rapidly under tap water. 1ml of 4% potassium sodium tartarate was added and the volume was adjusted to 12 ml with distilled water. A blank containing 1 litre of distilled water and 1 ml of dinitrosalicylic acid was similarly prepared. The optical density of the sample was read against the blank in a spectrophotometer at 540nm. The concentration of the reducing sugar in the supernatant was estimated from a standard glucose curve.

**Results and Discussion**

**Effect of process parameters on L-methionine production**

The effect of 11 process parameters such as plantain as carbon source, groundnut as nitrogen source, $CaCO_3$, $K_2HPO_4$, $KH_2PO_4$, biotin, $MgSO_4.7H_2O$, inoculum size, agitation speed, volume ratio of medium/fermenter and pH on L-methionine production by *C.glutamicum* were examined. In this work, 12 experimental runs were carried out to screen 11 parameters using Plackett-Burman design. The low and high levels of these parameters are used in PBD were shown in Table.1. The PBD matrix for influences of 11 parameters on L-methionine production and their responses are shown in Table.2.

Results from Table.2 indicated that highest concentration of L-methionine (4.5 g/L) achieved in run no.8 with the composition: plantain as carbon source- 25 g/L, groundnut as nitrogen source-15 g/L, $CaCO_3$- 25 g/L, $K_2HPO_4$- 0.5 g/L, $KH_2PO_4$- 0.5 g/L, biotin- 75 µg/L, $MgSO_4.7H_2O$- 2 g/L, inoculum size- 3 ml, agitation speed- 200 rpm, volume ratio of medium/fermenter- 35 %mL//mL and pH- 6.00 and the lowest concentration of L-methionine (1.6 g/L) achieved in run no.10 with the composition: plantain as carbon source-15 g/L, groundnut as nitrogen source-5 g/L, $CaCO_3$- 15 g/L, $K_2HPO_4$- 1 g/L, $KH_2PO_4$- 0.5 g/L, biotin- 125 µg/L, $MgSO_4.7H_2O$- 2 g/L, inoculum size- 3 ml, agitation speed- 200 rpm, volume ratio of medium/fermenter- 35 %mL//mL and pH- 8.00.

ANOVA of PBD results (Table.3) suggested that only 5 variables namely plantain as carbon source, groundnut as nitrogen source, $CaCO_3$, $MgSO_4.7H_2O$ and $KH_2PO_4$ had shown significant effect ($p<0.05$) on L-methionine production. The

remaining 6 variables not had shown significant contribution (p>0.05) to L-methionine production. There is a close agreement between experimental and theoretical values of L-methionine. The $R^2$ value of 0.99 proved that PBD model was significant in estimating the effects of variables on L-methionine production by *C.glutamicum*.

The Pareto chart (Fig.1) reveals the order of significance of parameters affecting L-methionine production and shows an easy way to view the results achieved in PBD experiment. From Fig.1 the order of most significant variables was shown as plantain as carbon source (A), groundnut as nitrogen source (B), $CaCO_3$ (C), $MgSO_4.7H_2O$ (G)

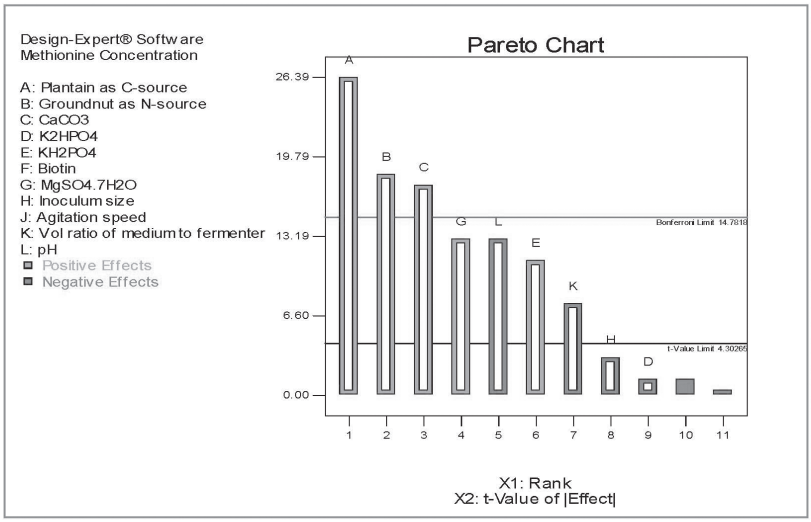


Fig.1 Pareto Chart for analysis of fermentation variables used in Plackett-Burman Design for L-Methionine production by *C.glutamicum*

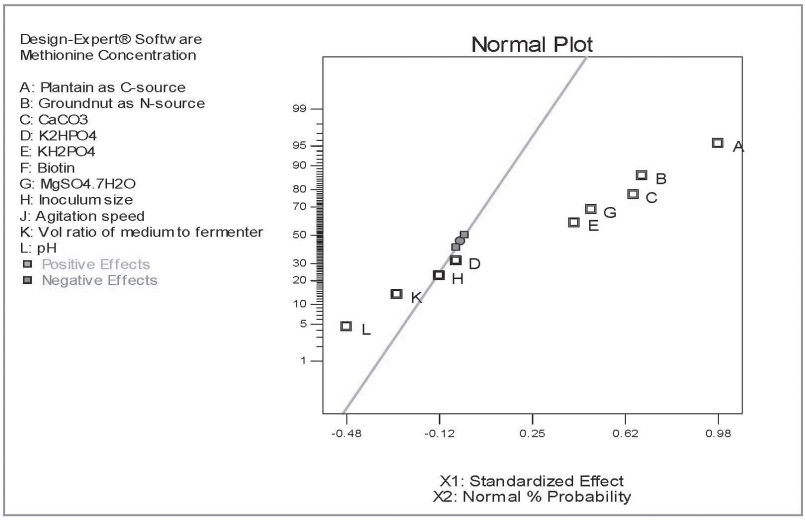


Fig. 2 Normal plot for estimation of effects for fermentation variables for the production of L-Methionine by *C.glutamicum*

and $KH_2PO_4$ (E) and remaining six variables had not shown a significant effect on L-methionine production.

Linear equations representing L-methionine production to process parameters (input variables) shown by PBD model as follows

L-methionine yield = 3.10 + 0.49*plantain as carbon source + 0.34*groundnut as nitrogen source + 0.32* $CaCO_3$ -0.025* $K_2HPO_4$ + 0.2* $KH_2PO_4$ + 0.24* $MgSO_4.7H_2O$ – 0.05* inoculum size – 0.14* volume ratio of medium to fermenter – 0.24* pH + 0.837* $MgSO_4.7H_2O$ + 0*agitation speed

Fig.2 reveals normal plot for L-methionine production to estimate the significant factors. In the normal plot of effects, the points which do not exist near the line are significant. Important effects are far away from the fitted line than unimportant effects. Unimportant effects tend to be smaller and centered on zero. In this case, important effects are plantain as carbon source, groundnut as nitrogen source, $CaCO_3$, $MgSO_4.7H_2O$ and $KH_2PO_4$ for L-methionine production by *C.glutamicum*.

**Optimization of process parameters for maximizing L-methionine production**

Fig.3 shows the ramps of PBD model estimates the optimum values of input process parameters for maximum production of L-methionine. As per the PBD model, the optimum values of input parameters were predicted as plantain as carbon source- 18.25 g/L , groundnut as nitrogen source- 14.1 g/L, $CaCO_3$- 9.25 g/L , $K_2HPO_4$-0 g/L , $KH_2PO_4$- 0.57 g/l , biotin- has no effect, $MgSO_4.7H_2O$- 1.9 g/L , inoculum size- 1 mL , agitation speed- has no effect , volume ratio of medium/fermenter- 31.5 and pH- 7.0. Experiments were carried out with optimum values and obtained the L-methionine production as 5.6 g/L. In comparison with unoptimized medium 24% increase in L-methionine yield was obtained.

In Plackett-Burman screening plantain and groundnut were two of most significant variables on L-methionine production and in the earlier

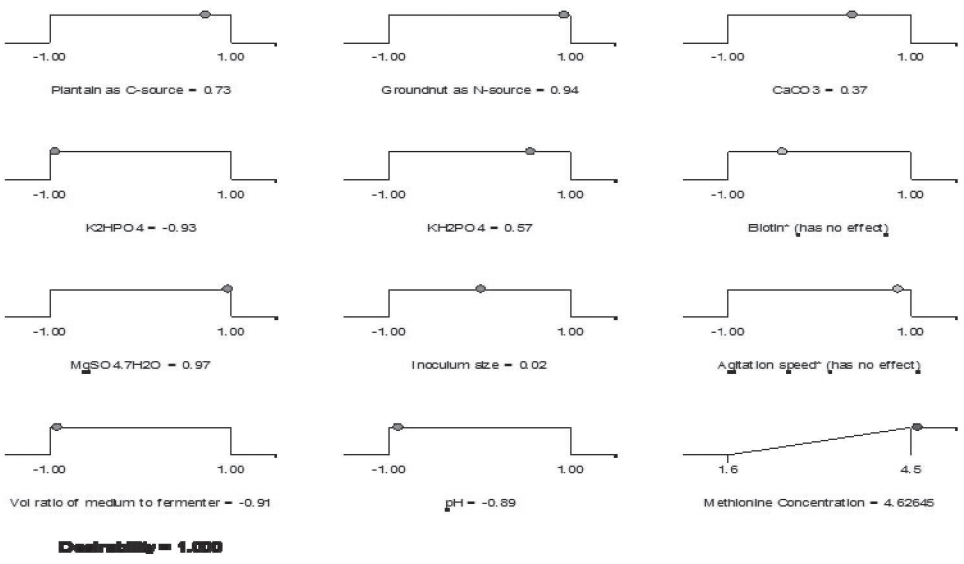


**Fig.3 :** Ramps of PBD model showing optimized values of input fermentation variables for maximum production of L-Methionine by *C. glutamicum*

Plackett-Burman design for L-methionine production

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Table.1 Low and high levels of nutrient (chemical) and physical process variables used in Plackett-Burman design for the production of L-Methionine by *C.glutamicum*

| S. No | Nutrient and physical variables with code | Minimum value (-1) | Maximum value (+1) | Units |
|---|---|---|---|---|
| 1 | Plantain as C-source (A) | 15 | 25 | g/L |
| 2 | Groundnut as N-source (B) | 5 | 15 | g/L |
| 3 | $CaCO_3$ (C) | 15 | 25 | g/L |
| 4 | $K_2HPO_4$ (D) | 0.5 | 1 | g/L |
| 5 | $KH_2PO_4$ (E) | 0.5 | 1 | g/L |
| 6 | Biotin (F) | 75 | 125 | µg/L |
| 7 | $MgSO_4.7H_2O$ (G) | 0.5 | 2 | g/L |
| 8 | Inoculum size (H) | 3 | 5 | ml |
| 9 | Agitation speed (I) | 160 | 200 | rpm |
| 10 | Volume ration medium/fermenter (J) | 25 | 35 | %ml/ml |
| 11 | pH (K) | 6 | 8 | - |

Table.2 Plackett-Burman Design matrix for L-Methionine production by *C. glutamicum* with experimental and predicted values.

| Run No. | A (g/L) | B (g/L) | C (g/L) | D (g/L) | E (g/L) | F (µg/L) | G (g/L) | H (mL) | I (rpm) | J (% ml/ml) | K (-) | L-methionine production (g/L) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Experimental | Predicted |
| 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 3.5 | 3.51 |
| 2 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | 3.7 | 3.69 |
| 3 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 2 | 1.99 |
| 4 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 3.9 | 3.89 |
| 5 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 3 | 2.99 |
| 6 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | 3.4 | 3.41 |
| 7 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 3 | 3.01 |
| 8 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 4.5 | 4.51 |
| 9 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 3.7 | 3.69 |
| 10 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | 1.6 | 1.61 |
| 11 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | 2.6 | 2.61 |
| 12 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | 2.4 | 2.39 |

works plantain and groundnut were reported by (12) using *Bacillus cereus* S8 from agricultural products and produced 2.05 mg/ml of methionine in submerged fermentation. In 2008, Adoki used plantain waste as carbon source for yeast growth and protein production by *Candida* species (13).

Another significant variable $MgSO_4.7H_2O$ has a positive impact on L-Methionine production, this is mostly due to methionine is sulphur containing amino acid addition of $MgSO_4.7H_2O$ to the medium improved the growth and L-methionine production and many workers (Kase et al. 1975, Banik et al.

Table.3 Analysis of variance (ANOVA) of PBD for L-Methionine production by *C.glutamicum*

| Source | Standard effects | Sum of squares | df | Mean Square | % Contribution | F-Value | P-value (Prob>F) |
|---|---|---|---|---|---|---|---|
| Model | ---- | 7.79 | 11 | 0.78 | …… | 934.60 | 0.0255* |
| Plantain (A) | 0.98 | 2.90 | 1 | 2.90 | 37.24 | 3481.00 | 0.0108* |
| Groundnut (B) | 0.68 | 1.40 | 1 | 1.40 | 17.98 | 1681.00 | 0.0155* |
| $CaCO_3$ (C) | 0.65 | 1.27 | 1 | 1.27 | 16.27 | 1521.00 | 0.0163* |
| $K_2HPO_4$ (D) | -0.05 | 7.5E-003 | 1 | 7.5E-003 | 0.096 | 9.00 | 0.2048 |
| $KH_2PO_4$ (E) | 0.42 | 0.52 | 1 | 0.52 | 6.69 | 625.00 | 0.0255* |
| Biotin (F) | -0.05 | 7.5E-003 | 1 | 7.5E-003 | 0.096 | 9.00 | 0.2048 |
| $MgSO_4.7H_2O$ (G) | 0.48 | 0.7 | 1 | 0.7 | 9.00 | 841.00 | 0.0255* |
| Inoculum size (H) | -0.12 | 0.041 | 1 | 0.041 | 0.52 | 49.00 | **0.0219*** |
| Agitation speed (I) | -0.017 | | 1 | 8.22E-003 | 0.011 | 1.00 | 0.4562 |
| Volume ration medium/fermenter (J) | -0.28 | 0.24 | 1 | 0.24 | 3.09 | 289.00 | 0.0903 |
| pH (K) | -0.48 | 0.70 | 1 | 0.7 | 9.00 | 841.00 | **0.0374*** |
| Residual | --- | 8.33E-04 | 1 | 8.33E-04 | --- | --- | 0.0219 |
| Cor Total | --- | ------ | 12 | --- | --- | --- | --- |

*Indicates significant variables for which (P<0.05) and * with bold p-values indicates significant but with negative effect and these were discarded in PBD model.

1975 and Tani et al. 1988) have been used $MgSO_4.7H_2O$ in their medium for production of L-methionine (14, 15 and 16).

Calcium carbonate is also one of significant variable for maximum production of L-methionine. It balances the pH of fermentation broth by eliminating lag phase of cell growth thus reduction in fermentation time. As nutrients are consumed and converted into products during fermentation process, the pH changes significantly in the absence of suitable control mechanism. In order to keep optimal pH, reagents such as calcium carbonate should be added to the fermentation medium at the starting of the fermentation [17]. The other variable $KH_2PO_4$ also affects the production of L-methionine. Due to the potential applications of methionine in feed, food, pharmaceutical and health industry, the results presented here are promising and can be used as insights to enhance production of L-methionine by fermentation at industrial level.

## Conclusions

The Plackett-Burman Design was efficiently used for screening of process parameters for L-methionine production by *C.glutamicum*. Results of PBD confirmed that five variables such as plantain as carbon source, groundnut as nitrogen source, $CaCO_3$, $MgSO_4.7H_2O$ and $KH_2PO_4$ were found to be most significant on L-methionine production by *C.glutamicum*. And the remaining six variables had not shown a significant effect on L-methionine production. In comparison with the unoptimized medium, 24% increases in L-methionine production was obtained from the optimized medium and L-methionine production was found to be 5.6 g/L. Owing to the potential

applications of L-methionine in feed and food industries, results presented here are very promising and provides new insights for enhancing L-methionine production at industrial level.

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# First week of social lockdown versus medical care against COVID-19 - with special reference to India

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**Abstract**

The disease caused by Coronavirus (CoV) is called as Coronavirus Disease (COVID-19). It became pandemic in 210 countries and rolling data indicate that 2,606,635individuals are infected with the deadly pandemic disease and 713,812 have recovered, while, 182,114 have lost their lives. Although, few drugs namely Chloroquine, Hydroxychloroquine and Azithromycin, Tocilizumab, Lopinavir; Ritonavir and Tocilizumab are recommended on contextual basis for the treatment of COVID-19, the world is still waiting to come up with a specific vaccine or medicine to combat the disease. Without a specific medicine, countries such as Italy, USA, Spain and France, withthe most advanced health care systems are unsuccessful to control both the infection and death rate under COVID-19 infection. India being the world's 2nd largest populous country, where, the health care system is under developed, with major portion of people live less-hygienic life, has contracted the disease at a slower rate as compared to the above countries. India has achieved this through employing social distancing by following a strict lockdown. This article is focused on social distancing of India and the kind of social interaction that can be adapted by people during lockdown.

**Keywords:** COVID-19;coronavirus, lockdown; India; pandemic spreading;socio-clinical management; social distancing.

## Introduction

Coronavirus (CoV) disease (COVID-19) is the greatest pandemic experienced by the world in which 210 countries are affected as of now (1). The infectious disease is caused by a new strain of the CoV for which exact medicines are unavailable for the treatment (2, 3). The disease is diagnosed mainly by dry cough, fever, tiredness and difficulty in breathingdue to accumulation of cough and subsequent fibrosis formation in alveolar region of lungs (4). The morbidity rate by the disease is found to be very high in elderly and in patients with co-morbidity and it may be due to their compromised immune system (5), (6-8). The disease can lead to mortality due to high fibrosis in the gas exchange part of the lungs that lead to respiratory choke. Therefore, the present disease resemblance with SARS and MERS The present virus has also found to have similarity with CoVs that cases SARS and MERS (7), (9-11). Structural analyses have confirmed that the present CoV i.e. CoV-19 is closely associated to two bat-derived SARS-like coronaviruses namely bat-SL-Cov-ZC45 and bat-SL-Cov-ZXC21, isolated in 2018 from Zhoushan, eastern China. CoV-19 has 79% genetic similarity with SARS-CoV and 50% with MERS-CoV indicate that it is a mutated form of the previous two (12, 13).

Specific medicine or vaccines to combat COVID-19 is not available although few drugs namely Hydroxychloroquine and azithromycin

(14), Lopinavir; Ritonavir (Remdesivir (GS-5734) (8), (15-21), Tocilizumab, corticosteroids (22, 23), certain nucleotide as inhibitors (24), COVID-19 protease inhibitor (25) are repurposed to be used against CoV-19 without much clinical experiences.

On the other hand, a country like India being the 2nd largest populous country, where, the health care system is severely suffering to advance in infrastructure (0.7 hospital beds per 1000 people), medical equipments and doctors (doctor: population ratio is 1:1800 instead of 1:1000), has contracted the disease with a slower rate. It remained interesting for other nations to know the reason behind it, because, a large portion of Indian population suffer from unhygienic life style.Two important key factors are believed to control the infection rate in India. 1) By adopting social distancing via social lockdown and 2) cleaning hands frequently using soap or alcohol based sterilizers. On the other hand, New Delhi government was supposed to did a lot of home work to incline the mind of its 1.3. billion people, despite few mismanagements were observed during the lockdown. All authentic news sources such as Science magazine, Reuters, BBC news, Times, Hindustan India, India Today, WHO etc. along with scientific literatures were carefully analysed the stricter social lockdown in India and its impact on prevention of COVID-19. Also, suggestions are places how social interactions can be done during social lockdown without physical meeting. This may provide outlook to Indian citizens not feel stressed during the long lockdown period.

**Infection of COVID-19 and its outbreak**

All strains of CoVsare not virulentto cause the disease (26, 27). CoVs are with single-stranded RNA genomes (28, 29) and out of its 7 strains, three, namely, CoV i.e. SARS-CoV, MERS-CoV and SARS-CoV-19 (COVID-19) are found to be virulent and can be fatal (30). The other four, namely HKU1, NL63, OC43 and 229E CoVs are not that much fatal and only yieldsmild clinical symptoms in patients (31). CoV-19 binds to the ACE2 receptor in human respiratory cells and attacks using its protein called as “S” which is cleaved into two, namely S1 and S2 that mediate the viral fusion into the host cell (32, 31). Finally, it moves from upper respiratory passages to lower respiratory passages and finally reached to alveoli. At this gas exchanging part, it makes huge fibrosis leading to respiratory choke and death of the patient (33).The first recognized case of severe clinical sickness caused by a CoV was found in 2003 in China (SARS) that took 774 lives (34-37). The second outbreak was reported in 2012 in Saudi Arabia (MERS) till February 2018, 2182 cases with 779 death sare reported by MERS in 27 countries. Saudi Arabia alone experienced 1807 infection and 705 deaths in MERS. The third case is the CoV-19 that infected 24,16,135 individuals as of 22.04.2020, out of which 6,32,983,279 have recovered, and 1,65,939 did not survive the present CoV-19 outbreak.

**Clinical management of COVID-19**

Although some of the existing drugs are repurposed to combat COVID-19, WHO has been repeatedly suggesting for social distancing for avoiding close contact (1 meter or 3 feet) with people who are unwell.Because the disease is believed to spread primarily through contact with an infected person via their cough or sneeze. It also can infect when a person touches surfaces or objects that carry the virus. Because from hand, it then infect when one touches eyes, nose, or mouth. Organisations such as FDA, CDC and WHO have repurposed few of the drugs namely chloroquine or hydroxychloroquine (both are antimalarial drug), lopinavir; ritonavir which is an HIV protease inhibitors, azithromycin which is a macrolide antibacterial medicine, tocilizumab that is an interleukin-6 receptor-inhibiting monoclonal antibody, COVID-19 convalescent plasma (Plasma collected from persons who have recovered from COVID-19 that may contain antibodies to CoV-19) and corticosteroid therapy, albeit above drugs have their limitations, risk factors, as well as context dependent prescription is required (23).Patients with co-morbidity such as diabetes, cardiac issues cannot consume above drugs as it may lead to serious complications. So, each

drug is focusing to a particular aspect of the clinical consequences in COVID-19 patients (38). Therefore, social lockdown is the only preventive measures found to be effective in the present condition (33).

**Social lockdownand its scientific background**

Social lockdown is direction given by concerned authorities for avoiding inter-individual physical contacts, to avoid outer environment and to stay indoors. Minimal movement of general public under emergencies and services such as medical care, food security, general security and medicine supply chain are usually allowed. It is therefore slightly less stringent than curfew. Under high emergency of social lockdown, above supplies are also not given if the time period is limited for few hours or days. The central objective of this social movement under government direction is to for bid two people from different family or nearby inhabitant to come close contact with each other (Fig. 1).

Due to slow infection rate under social distancing, it also contribute to a) prevent huge load of COVID-19 patients on healthcare system at a particular time point, b) restrict community infection (stage III) and c) to allow hospitals to provide better health care to the infected persons.

It was noticed that article on "social lockdown" in PubMed in relation toCOVID-19 infection is very scanty. One article published by (Doremalen, N. et al.) (39) discussed about the potential sources of pandemic of this disease in China. They have noticed that people without travel history can contract the disease if social distancing in not maintained (39).

***International status on social lockdown:*** China has found to implement social lockdown in last week of January 2020 in Wuhan city that acts as the epicentre COVID-19 outbreak. Then it followed gradual social lockdown in Beijing where buses and carswere allowed to run but domestic flights and trains were cancelled. But China kept Wuhan city completed isolated for which it has experienced low infection rate as compared to other nations (40). Social lockdown in China had covered about 760 million people (41). However, in India, all of its 1.3 billion people were covered under social lockdown (41, Fig. 1a). Italy was the second country to follow social lock down but with not a very stringent way. Italy had declared a lockdown on 21st February 2020 in north Italy covering only 50,000 people which was then extended to its whole country under on 9th March 2020 but allowed public transport to run partially for which the country could not control the disease. Also mainly elders have contracted the disease in Italy that leads to high mortality under COVID-19 (42, Fig. 1b).

USA is now the highest sufferers in COVID-19. The reason is attributed to a) its high percentage of migrants as compared to the native Americans b) high rate of clinical diagnosis for COVID-19 and c) late and casual steps for observing social lockdown (43). Trump government has explained that "The better you do, the faster this whole nightmare will end; therefore, we will be extending our guidelines to April 30th to slow the spread" (43). The Director of NIH has also recommended USA people to adopt social distancing in a stricter way (44, Fig. 1c).

Bangladesh has implemented the act of social distancing 25th March 2020 the country was following a 10-day "holiday," after this date. On 20th March 2020, this country had already warned its citizens to implement social lockdown for COVID-19 outbreak, so people were able to travel to their native before lockdown started (45, Fig. 1d). Sri Lanka with ~ 20 million populations had initially declared holiday and urged its citizens to observe social distancing (46). Then the government had officially declared lockdown in its entire nation (47). Pakistan had formally declared lockdown in the entire country on 23rd March 2020 with an advisory to its citizens to follow social distancing with all possible measures. Later on the country had taken special measures to de-containment various infected places like Sindh, where the lockdown was later made the most severe (48). The Pakistan government had allowedall passenger trainsto move for six more days to allow people to reach their destination.

Therefore, people were able to reach their native before full lockdown was implemented fully (49, Fig 1e).

India is the 2nd most populous nation can only afford 0.7 hospital beds per 1000 people, although many alike countries such as Afghanistan (0.5), Bangladesh (0.8), Cambodia (0.8), Ethiopia (0.3), Ghana (0.9), Guinea (0.3), Madagaskar (0.2), Mozambique (0.7), Nigeria (0.5), Pakistan (0.6), Sudan (0.8), Uganda (0.5) and Yemen (0.7) are on the board to provide <1 hospital beds per


**Fig. 1.** The lockdown to combat COVID-19 across the globe. a) Wuhan city China in January 2020, b) social distancing in a lift in Italy, c) in USA street, d) in Bangladesh, e) in Pakistan (movement from Karachi to destination native), f) in India, rules are made near shopkeeper to sell those, who maintains social distancing (g). (source google.com from respective news sources)

**Fig. 2.** Large scale migration of people to their native after declaration of lockdown in India. Large scale inter-state migration in India after the national lockdown was declared on 24th March 2020. False social media news about special interstate bus services also tempted people to aggregate at many bus stands across the country such as AnandVihar, NewDelhi, that made a harsh condition to government (source,51)

(their) 1000 citizens (50, Table 1, 33). It has only estimated 48,000 ventilators available for its whole 1.3 billion population (51). As per the last updates, doctor: population ratioin India is 1:1800 instead of 1:1000. Although, some claims India has the required ratio already from 2018 if of all AYUSH doctors are included (52). On the other hand, a large portion of Indian population lives an unhygienic life style and contracts the infectious and endemic associated diseases (53) and it could welcome to infect the community by endemics and subsequent outbreak (54). So, India could be suspected as one of the finesthome for the outbreak of the pandemic COVID-19 (33).

Owing to this view in mind, the federal government in India had taken a very early and strict (some called it as harsh) decision for the entire nation to declare lockdown. On 11th March 2020, WHO declared an emergency on the outbreak of COVID-19 and appeals all nations to take stringent actions to protect their citizens.In India, the marked beginning of infection by CoV-19 was started after 9th March 2020 (33). On 24th March 2020, India had declared the entire nation to follow lockdown for 21 days which was then extended up to 3rd May 2020. Because India had received expected success by lockdown. The infection in pandemic was slower in India as compared to other countries. As of 5th April 2020, 6:00 pm, according to the Ministry of Health & Family Welfare, New Delhi, a total of 3577 COVID-19 cases including 65 foreign nationals, 274 recovered, 83 deaths, and 1 migrated, have been identified in its 29 states/union territories. However, many also opined that a low infection number could be due to lack of massive and random medical diagnosis and hot and humid climate of India disallowing the virus to propagate and infect. Although scientific fidelity of these facts are not yet proved, at least the lockdown time had allowed all the non-resistant asymptomatic infected patients to exhibit clinical symptoms in time, and it was still very less after 14 days of lockdown was over on 6th April 2020 (Fig. 1f, g). On the other hand, many also put statements against the lockdown byIndian government, describing it as harsh, intensive and mismanaged (46,55) but the government had archived the successby employing many strategic plans such as “Janata Curfew”, ring the bell, lightening the candle, torch and mobile lights, that encouraged Indian to follow the social move of lockdown (7, 10,40, 56, 57). Except intelligence failure of Tablighi Jamaat event, a Muslim religious observed at Nizamuddin in Delhi’s where about 8,000 indigenous and foreign Muslims were gathered. The people attended the event from different countries and almost from all states of India, now contribute has been contributing to 30% of the total COVID-19 positive cases in India, and counting is on (57, 33).

**Adverse effects ofsocial lockdown in India and its management:** The social move in India for lockdown was criticized by many, describing it asIndia is unable to provide healthcare to its citizens as compared to countries like China and USA (58, 46). On the other hand, it had a massive negative impact on society especially on weaker section population especially on the daily wages, interstate migrant workers and small scale vendors. Many cities such as Mumbai, Kolkata, Chennai, Bengaluru have experienced huge economic loss (Table 2, 3 and 4). Large scale intra and interstate migrants were found to walk to their home due to lack of transport services (59), although it was considered as against the legal move and action was urged to provide food and shelters to such migrants (60, 61, Fig. 2, 3). Indian Government had declared a $22.6 billion economic package for people who were affected by lockdown (55). With the help of state governments, NGOs, local bodies the Federal Government had handled the situation (62, Fig. 2, 3) and at least 100 to 200 daily wages per one village cluster (called as Gram Panchayat) have been provided with food and shelter for the entire lockdown period (62, 63).Few state governments such as Odisha (¹ 3,000 to its 65, 000 vendors, 64), and New Delhi (¹ 5,000 INR to auto rickshaw and taxi drivers) provided financial assistance. Toll free telephone numbers were issues to take care of COVID-19 patientsfree of cost (65). The World Bank had announced $1 billion support to India


**Fig. 3.** Free foods and shelters are provided to the daily wages, migrant workers in India. Photo was taken from Sambalpur (a), Odisha and Mahanga (b), Odisha state (sources New Indian Express and Livemeant.org), c). People on social distancing to receive food in Central Reserve Police Force during the lockdown in Chennai, on April 1, 2020(source Indian Today, Hindustan Times, The Hindu, New18, India).

**Fig.4.** Effects of lockdown on human on road in different cities across world. The cleaner air could able to show the real landscape of different cities of the planet on 25th March 2020 (Source CNN, 71).

**Fig. 5.** Effects of 21 days lockdown on the sparkling Yamuna river water, New Delhi in first week of lockdown. It may be due to the shutdown of industries and lack of release of their effluents into it in Delhi (source, Time of India, 72).

(67), while, USA had declared a $2.9 million financial assistance to combat COVID-19 (68). Subsequently, each state government were provided with a financial assistance of ¹ 11,092 from the Federal Government to combat COVID-19 outbreak (69). Therefore the social lockdown move was successful in India as opined by WHO(70). Although, systematic, random and large-scale clinical diagnosis for COIVD-19 positive cases in India is advised (71).

***Ecologicaleffects of lockdown in India:*** As a result of lockdown and shutdown in some places in India, and abroad air and water pollution was decreased (Fig. 4). The reason is attributed to reduction in emission from fossil fuels from different sectors (72).The average $NO_2$ and $CO_2$ in major cities such as Mumbai, Pune and Ahmedabad was reduced in March 2020 as compared March 2019. The sky of New Delhi was clearly visible than four months ago. Also the lockdown effects were very clear on water bodies. Some of the river water bodies are found to exhibit not ever seen glittering scenery. The Yamuna river and its sky in New Delhi can be considered as an active example, in which, people have not ever observed the before seen sparkling Yamuna river water and the blue sky canopy over it (Fig. 5). It may be due to the shutdown of industries in New Delhi and lack of release of their effluents into its (73, 74).

As per many, social lockdown really provides opportunity to spend time with family members. Therefore, it meant for real social interaction among family and friend but without physical meeting. A glimpse is given how to spend time happily under social lock down period.

**Social interaction during social lockdown**

Across the world people are now facing a very difficult pandemic situation due to a novel corona virus. In these circumstances most of the nations declared the only method to get rid over from this pandemic is social distancing and social lockdown for their citizens.In India, 1.3 billion people have been locked down for one month and in second phase lock down from 14th April night to curb the spread of the coronavirus. Lockdowns, quarantines and extreme forms of physical distancing work are curbing the spread of Covid-19. Hence the first objective of any response and the government any where must be to protect lives, and that means averting the collapse of the health care system. Hospitals are the last line of defence when their capacity to handle emergencies is over whelmed. Social distancing and lockdown are the most effective way of fighting against corona virus. Nevertheless, people have to understand that social distancing does not mean ending social interaction. Minimising social interactions does not mean that one can decrease emotional attachment or ending the social interaction. The abridged social interaction with friends, relatives and neighbour without physical interaction is a difficult but possible job.In indicates that navigating this new situation in (a new) world of social limitations is very challenging. It is the high time to transform the life in a better way during this lock down. So people should think how to use this opportunities in productive way rather than focuses on anxiety/stress and feel depression (75, 76). There are two ways to connect with families, relatives, friends and neighbour, 1) Intra-family interaction and 2) Inter-families interaction. Intra-family interaction is the interaction between family members and Inter-familiesinteraction, which is between family to family interactions with social distancing mode and feels joyful in the life and also support the world to overcome from this panic situation. Working from home is the best concept, but rest time can be managed in a constructive way. Since adults can manage their time, more emphasis is given how to manage children during long term lockdown.

**Intra-family interaction**

Interaction is the mode of develop the hope in life and enlighten the thoughts. In 21stcentury, people are extremely busy in their work schedule and business to earn money, name and fame. Contemporarily, people have no time to spend the quality time with family members, friends, relatives and neighbour. It is becauseallmost all are running behind money, power and position. No one cares for the social relationship and

emotional bonding. Interactions incorporate sectors give experience to individuals with members of their social and global networks (78). Social relationship is an important part to develop cognitive elements (79). Human beings are advanced in science and technology and reached in a place, where a good social relation is just a dream. Joint families converted to nuclear families, and in nuclear family, parents have been not giving much time to their children due to work pressure. So, this social lockdown is the golden opportunity to enjoy quality time which human beings have forgotten to realise and maintain in long term basis. Present time is to give new bond in life to all existing social relationships. Citizens are restricted in their house to maintain social distancing but are not alone. They are staying safely with their family members in their respective place and this is the high time people may uphold hobbies such as gardening, music and spending quality time with their families during lockdown to take the advantages of social lockdown. Lockdown may be enjoyed in many ways.Some of the interfamily interactions are suggested below.

***Energetic, quality time with family:*** It's very difficult to spent time with family when all parks, malls, visiting places are closed. So keeping dynamic members of family active is a very difficult task. Most important factor is maintaining both physical and mental 'health' of family member (80,81). It will build up their immunity and mental power and will make them free from all the negativity, anxiety, and stress during lockdown period. Regular family yoga, exercise in morning and/or evening is the key point. It positively benefits to an individual in countless ways, both physically and psychologically (82, 83). Therefore, integratinggood chunks of physical activity into their daily life schedule is important. As a rule, adults need at least60 minutes of "moderate to vigorous physical activity" almost every day, while children need30 minutes moderate physical activity every day. "Moderate to vigorous physical activity" makes the heart and lungs work harder than they normally do. Usually a good fast walk in treadmill or an activity such as jogging, running and cycling are few to name them. If space limits physical exercise, then Yoga: pranayam, breathing exercises and asanas, laughing exercise and meditation may be adapted. Breathing exercises take care of lungs, heart and build upimmunity. Laughing exercise and meditation balance the mind with calmness and provide peace and increases the positive aura and decrease the negativity from the mind like anxiety, fear, panic, stress, and tension(84,85).

***Revisiting interest and passions:*** Due to job pressure, people usually lost lots of hobbies, interest and passion they adapted during their childhood. This is the time to active their interest and passions. There are many passions such as painting, singing, dancing, cooking, gardening and reading can be revisited.

***Nurture the nature:*** Mother Nature always offerscomfort and peace to human like their biological mother does.Many-a-time human takesit for granted and does not look back to nurture nature, what she deserves. Small scale contribution such as gardening, watering the flower plants, sit in a balcony and observe the beautiful sounds of the birds and offer food to the animals and birds can lead to a massive change. Human need to regard nature as an infinite source of ecstasy, amidst the din of their hectic life to take them close to the almighty as nature is his art to soothe the human beings.

***Offering indoor games:*** To keep self and children sane over the period of long term lockdown it would not be easy. Tight work schedules disallow parents to spend time with their family including child. Escalating household chores cut through what little time they might manage to cook up. Kids, being little balls of curiosity, get bored of the same activity very quickly and are constantly looking for new and new challenges. And at this crucial point of time this often leaves parents clueless as to how to engage a child, when all the parks and outdoor games are closed due to social lock down. There's more to engaging children with meaningful activities than just ridding them of their boredom. Indoor game which parents can play with them

**Table** 1. Countries with number of hospital beds per 1000 people (Source World Bank, 2020)

| Country | Number of Beds | Country | Number of Beds | Country | Number of Beds | Country | Number of Beds | Country | Number of Beds |
|---|---|---|---|---|---|---|---|---|---|
| Afghanistan (2015) | 0.5 | Egypt, Arab Rep. (2014) | 1.6 | Lao PDR (2012) | 1.5 | Russian Federation (2013) | 8.2 | Portugal (2013) | 3.4 |
| Albania (2013) | 2.9 | Estonia (2015) | 5 | Latvia (2013) | 5.8 | Samoa (2005) | 1 | Qatar (2014) | 1.2 |
| Algeria (2015) | 1.9 | Ethiopia (2015) | 0.3 | Libya (2014) | 3.7 | San Marino (2012) | 3.8 | Romania (2013) | 6.3 |
| Andorra (2009) | 2.5 | Fiji (2011) | 2.3 | Lithuania (2013) | 7.3 | Lao PDR (2012) | 1.5 | Russian Federation (2013) | 8.2 |
| Antigua and Barbuda (2014) | 3.8 | Finland (2015) | 4.4 | Luxembourg (2015) | 4.8 | Latvia (2013) | 5.8 | Samoa (2005) | 1 |
| Argentina (2014) | 5 | France (2013) | 6.5 | Madagascar **(2010)** | 0.2 | Libya (2014) | 3 | San Marino (2012) | 3.8 |
| Armenia (2015) | 4.2 | Gabon (2010) | 6.3 | Malawi (2011) | 1.3 | Lithuania (2013) | 7.3 | Sao Tome and Principe (2011) | 2.9 |
| Australia (2014) | 3.8 | Georgia (2013) | 2.6 | Malaysia (2015) | 1.9 | Luxembourg (2015) | 4.8 | Saudi Arabia (2014) | 2.7 |
| Austria (2013) | 7.6 | Germany(2013) | 8.3 | Maldives (2009) | 4.3 | Madagascar (2010) | 0.2 | Serbia (2012) | 5.7 |
| Azerbaijan (2013) | 4.7 | Ghana (2011) | 0.9 | Malta (2014) | 4.7 | Malawi (2011) | 1.3 | Seychelles(2011 | 3.6 |
| Bahamas, (2013) | 2.9 | Greece (2015) | 4.3 | Marshall Islands (2010) | 2.7 | Malaysia (2015) | 1.9 | Singapore (2015) | 2.4 |
| Bahrain (2014) | 2 | Greenland (1970) | 14.4 | Mauritius (2011) | 3.4 | Maldives (2009) | 4.3 | Slovak Republic (2015) | 5.8 |
| Bangladesh (2015) | 0.8 | Grenada(2014) | 3.7 | Mexico (2015) | 1.5 | Malta (2014) | 4.7 | Slovenia (2013) | 4.6 |
| Barbados (2014) | 5.8 | **Guinea (2011)** | 0.3 | Moldova (2013) | 5.8 | Marshall Islands (2010) | 2.7 | Solomon Islands (2012) | 1.4 |
| Belarus (2013) | 11 | Guinea-Bissau (2009) | 1 | Monaco (2012) | 13.8 | Mauritius (2011) | 3.4 | Somalia (2014) | 0.9 |
| Belgium (2014) | 6.2 | Guyana (2014) | 1.6 | Mongolia (2012) | 7 | Mexico (2015) | 1.5 | South Africa (2005) | 2.8 |
| Bermuda (1996) | 6.3 | Hong Kong SAR, China (1985) | 4.9 | Montenegro (2012) | 4 | Moldova (2013) | 5.8 | Spain (2013) | 3 |
| Bhutan (2012) | 1.7 | Hungary (2013) | 7 | Morocco (2014) | 1.1 | Monaco (2012) | 13.8 | Sri Lanka (2012) | 3.6 |
| Bosnia and Herzegovina (2013) | 3.5 | Iceland (2014) | 3.2 | **Mozambique (2011)** | 0.7 | Mongolia (2012) | 7 | St. Kitts and Nevis (2012) | 2.3 |
| Brazil (2014) | 2.2 | India (2011) | 0.7 | Myanmar (2012) | 0.9 | Montenegro(2012) | 4 | St. Lucia (2013) | 1.3 |
| Brunei Darussalam (2015) | 2.7 | Indonesia (2015) | 1.2 | Namibia (2009) | 2.7 | Morocco (2014) | 1.1 | **Sudan (2013)** | 0.8 |
| Bulgaria (2013) | 6.8 | Iran, Islamic Rep. (2014) | 1.5 | Nauru (2010) | 5 | **Mozambique (2011)** | 0.7 | Suriname (2010) | 3.1 |
| Cambodia (2015) | 0.8 | Iraq (2014) | 1.4 | Nepal (2012) | 0.3 | Myanmar (2012) | 0.9 | Sweden (2013) | 2.6 |
| Cameroon (2010) | 1.3 | Ireland (2013) | 2.8 | Netherlands (2009) | 4.7 | Namibia (2009) | 2.7 | Switzerland (2013) | 4.7 |
| Canada (2012) | 2.7 | Israel (2013) | 3.1 | New Zealand (2013) | 2.8 | Nauru (2010) | 5 | Syrian Arab Republic (2014) | 1.5 |
| Cayman Islands (1990) | 3 | Italy (2012) | 3.4 | **Nigeria (2004)** | 0.5 | Nepal (2012) | 0.3 | Tajikistan (2013) | 4.8 |
| Central African Republic (2011) | 1 | Jamaica (2013) | 1.7 | North Macedonia (2013) | 4.4 | Netherlands (2009) | 4.7 | **Tanzania (2010)** | 0.7 |
| Channel Islands (1981) | 10.6 | Japan (2012) | 13.4 | Norway (2013) | 3.9 | New Zealand (2013) | 2.8 | Thailand (2010) | 2.1 |
| Chile (2013) | 2.2 | Jordan (2015) | 1.4 | Oman (2014) | 1.6 | **Nigeria (2004)** | 0.5 | Timor-Leste (2010) | 5.9 |
| China (2012) | 4.2 | Kazakhstan (2013) | 6.7 | **Pakistan (2014)** | 0.6 | North Macedonia (2013) | 4.4 | Tonga (2010) | 2.6 |
| Colombia (2014) | 1.5 | Kenya (2010) | 1.4 | Palau (2010) | 4.8 | Norway (2013) | 3.9 | Trinidad and Tobago (2014) | 3 |
| Comoros (2010) | 2.2 | Korea, Dem. People's Rep. (2012) | 13.2 | Panama (2013) | 2.3 | Oman (2014) | 1.6 | Tunisia (2015) | 2.3 |
| Costa Rica (2015) | 1.2 | Korea, Rep. (2015) | 11.5 | Paraguay (2011) | 1.3 | **Pakistan (2014)** | 0.6 | Turkey (2013) | 2.7 |
| Croatia (2015) | 5.6 | Kuwait (2014) | 2 | Peru (2014) | 1.6 | Palau (2010) | 4.8 | Turkmenistan(2013 | 7.4 |
| Cuba (2014) | 5.2 | Kyrgyz Republic(2013) | 4.5 | Philippines (2011) | 1 | Panama (2013) | 2.3 | Tuvalu (2001) | 5.6 |
| Cyprus (2013) | 3.4 | Lao PDR(2012) | 1.5 | Poland (2013) | 6.5 | Paraguay (2011) | 1.3 | Uganda (2010) | 0.5 |
| Czech Republic (2015) | 6.5 | Latvia (2013) | 5.8 | Portugal (2013) | 3.4 | Peru (2014) | 1.6 | Ukraine (2013) | 8.8 |
| Denmark (2015) | 2.5 | Kuwait (2014) | 2 | Qatar (2014) | 1.2 | Philippines (2011) | 1 | United Arab Emirates (2013) | 1.2 |
| Dominica (2012) | 3.8 | Kyrgyz Republic(2013) | 4.5 | Romania (2013) | 6.3 | Poland (2013) | 6.5 | United Kingdom (2013) | 2.8 |
| United States (2013) | 2.9 | Virgin Islands (U.S.) (1998) | 18.7 | Uruguay (2014) | 2.8 | Yemen, Rep. (2014) | 0.7 | Uzbekistan (2013) | 4 |
| Zambia (2010) | 2 | Vietnam (2014) | 2.6 | Zimbabwe(2011) | 1.7 | World (average) | 2.7 | | |

are chess, carom, ludo, making puzzles, building blocks and many more mind teasing games, playing cards, hide and seek etc. This can be the way parents get engage or develop a tight parental relation with their children. This will give heart health as well as skill development benefits. Act of playing will disallow them not to feel bore and stay safe and not feel alone and remain inside, yet could be an additional mission of this social lock down.

***Teaching about moral value system and positive aura:*** Value is the amount of worth ascribed to something is prized or has merit. Values are the beliefs that each person considers are important for himself and possibly for humanity as whole. It is important that parents instil good values in their children. Values are very important in parenting since they deeply influence all behaviours and attitudes and affect our decisions and relationships. Adults play a very important role to strengthen the child's mind. Adults provide a frame work of 'storytelling' which enables the child to learn the value system. In contexts that are familiar and routinized the adult, one step ahead of the child, cues the child's responses. By providing ritualised dialogue and constraints through questioning and feedback to the child, the adult prepares the cognitive base on which language and knowledge is acquired (86-88). As a result they can feel safe andstay at home to learn the great things from their parents, and elders through the medium of story (89). Story and folktales as a focus for talk and interactive story telling plays an important part in helping

**Table 2.** Average decrease (negative values in table) in Change Over historical high (%) of footsteps counted in petrol pumps and banking sector in Delhi and Mumbai. (Data source Google foot prints).

| Day in first week of lock down | Delhi | | Mumbai | |
|---|---|---|---|---|
| | Banking | Petrol pump | Banking | Petrol pump |
| Monday | -65 | -94 | -78 | -42 |
| Tuesday | -64 | -96 | -86 | -57 |
| Wednesday | -52 | -93 | -81 | -74 |
| Tuesday | -33 | -93 | -69 | -78 |
| Friday | -66 | -97 | -75 | -78 |

**Table 3.** Average decrease or increase (negative and positive values respectively, in table) in Change Over historical high (%) of into grocery Shops at Delhi, Mumbai, Bengaluru and Kolkata in first week of lockdown. (Data source Google foot prints).

| Day in first week of lock down | Delhi | Mumbai | Bengaluru | Chennai | Kolkata |
|---|---|---|---|---|---|
| **Monday** | 3 | 19 | 73 | 284 | 39 |
| **Tuesday** | -22 | -24 | -31 | 200 | -38 |
| **Wednesday** | -16 | -44 | -15 | 61 | -40 |
| **Tuesday** | -10 | -24 | -14 | 58 | -38 |
| **Friday** | -16 | -33 | -38 | 73 | -50 |

**Table 4.** Average decrease or increase (negative values in table) in Change Over historical high (%) of into diagnostic centres at Delhi, Mumbai and Bengaluru in first week of lockdown. (Data source Google foot prints).

| Day in first week of lock down | Delhi | Mumbai | Bengaluru |
|---|---|---|---|
| Monday | -43 | -41 | -17 |
| Tuesday | -50 | -49 | -60 |
| Wednesday | -51 | - | -83 |
| Tuesday | -63 | -63 | -75 |
| Friday | -61 | -61 | -70 |

children to develop their expressive and learning the good thoughts and also take them away from feeling of stress and getting bored (90). If parents donot accept this responsibility, then the void may be filled by negative forces in the culture that do not support healthy morals and ethics for the families. At current time point, parents must teach values. For a value to be truly your own, you must act on it and your behaviour must reflect it not just verbally accept it or think that one should follow it. Teach the children values like kindness, creativity, honesty, punctuality, neatness, socialization time with peers or time with family, through beautiful short stories. The more aware parents are of their own values, the clearer they will be in expressing them and communicating them to other and their children. Time may be spend with grandparents to listen stories, tales and narratives and through which children can know the good characters and imagine them learn the values from that characters like,

- Acceptance: cordial to others whose ideas, background and practices differ from your own.
- Compassion, Cooperation and teamwork: understanding the suffering of others or self and wanting to do something about it and helping your family and friends, returning favours.
- Courage, and fairness: willingness to do difficult things, believing everyone deserves equal rights and to be treated with respect and acting in a just way, sharing appropriately.
- Charity, Gratitude, honesty, integrity: willingness to give resources, help or time to others, showing appreciation to others, letting loved ones know what you appreciate about them, being truthful and sincere, sticking to your moral and ethical principles and values, being considerate and treating others well.
- Politeness, respect, responsibility, self-control, and tolerance: persisting in a course of action, knowing to use of good manners, acting in socially acceptable ways, showing consideration for the worth of someone or something, being reliable in your obligations, staying in control of your words and behaviour, having a fair and objective attitude towards different opinions, beliefs or practices.
- Trustworthy (being truthful): reliably doing what is right even when it is difficult, being true to your word.

***House cleaning and cooking:*** As everyone is now practising social distancing, in line with government guidance, citizens need to clean their home on regular mode and sanitize with bleaching water or any other sanitizer. Amid COVID-19 fear, maid or other domestic help can be avoided to miscommunicate the disease. The burden of managing the household chores will not be complicated task, if handled together. Person need to juggle their work, kids, family, household

chores, everything together. Every family member should reflect in the daily chores. Division of labour may also be followed not as a rule but to adapt altruism. Collective efforts will not only help in managing the chores easy, but will also keep everybody busy. In order to make cooking fun and interactive, make it a family activity. Everyone may be asked to make a new dish at their own capacity or learning from YouTube or any other sources. And a score may be given to make minds competitive to discover new dishes.

***Watching television together:*** The COVID-19 outbreak has brought unparalleled change to lives across the world. As people stay indoors, work from home and look for ways to keep themselves engaged. And when, household works are over watching television is the most important medium to engage one self and feel relaxed in this critical time. Especially, many gyms, zumba and yoga institutions are providing online video services and can be learnt together. Many ethical stories such as Ramayan and Mahabharat in India, which children are not yet learnt, are replayed and must be shown to them.

***Up gradation of life:*** The present situation is now out of control. The only precaution of this condition is that to stay at home and use this social lockdown period productive rather stay at home with fear. Upgrading children how to plan for future is important. So studies, looking for career point, fix the aim and goal with proper counselling to children value the life. Lockdown period is to revisit and built the personality and focus on how to be a good human being. This is called as up gradation of life. One can ahead or in advance of his/her life in every prospect and feels hassle-free after this critical situation. So, one should make sure how to emerge with full of energy and skill to revert back to work for multi-fold productivity to compensate the loss during lockdown.

***Upgrade knowledge:*** **L**ong term social lockdown provides the time to contemplate and reflect back to upgrade with new knowledge. This can be done with professional interaction over telephone or internet. Tight work schedule does not provide time to upgrade with new knowledge. If this small objective is achieved at individual level, then a new world will be emerged after end of COVID-19 eradication. Knowledge may also be enhanced form online new books, novels, scientific magazine, good novels, story books, history, philosophy, traditional scriptures etc.

**Inter families interactions**

Social lockdown has limited movement and activities and essentially limit interactions with loved ones, relatives, neighbour, friends, which in turn can ensure a huge psychological pressure on a person. This is the eve of pre-summer vacation in many south Asian countries including India. Citizens usually plan for family gatherings, socialising with friends and perhaps participating in special religious activities. Sudden impacts of social lockdown may find themselves in an unprecedented and unimaginable situation with various uncertainties dwelling in minds. For decades, research has been increasingly demonstrating that social interaction significantly contributes to people's health and longevity (91,92). In addition, social interaction not only gives pleasure, but also influences long-term health in ways similar to adequate sleep, a good diet etc. Emotional support linked with social interaction helps to reduce the negative effect of stress and can cultivate a sense of meaning and purpose in life, while at the same time; it also lowers levels of anxiety and depression (93-95). Also, limited social interaction during long term lockdown may contribute to people experiencing loneliness, isolation and alienation.

***Social participation without physical interaction:*** It is broadly acknowledged that social relationships and its connection have powerful effects on physical and mental health. When investigators write about the impact of social relationships on health, many terms are used loosely and interchangeably including social networks, social ties and social integration. And these networks operate at the behavioural level through four primary pathways namely, (1) provision of social support, (2) social influence,

(3) on social engagement and attachment and (4) access to resources and material goods (96,97). In these four pathways, people can overcome with a great hope and maintain the social gap among each other. The first three modes can be achieved through social media. However, social media can act as a double-edged sword if used in improper way. In one hand, it can be a vital source of connection, for sharing information and social interaction which is more important than ever now that most of our physical interactions with our friends and family have stopped. On the other hand, currently, it is all too easy to fall into a deep, dark social media hole. Nonetheless, this is the high time to giving strength to the families, friends those are far away from their families and there are no ways to any kind of physical interaction which is prohibited under social lockdown. The only mode to interact with them is through telephone and social media. It is certainly true that during times of crisis, the inescapable cycle of news and avalanche of hot takes can leave one to feel utterly overwhelmed and exhausted. Oneneeds to find connections with other people wherever possible. Social media might become more important than ever in the coming weeks. And,all need to learn how to use it in a positive and helpful way. Positive engagement in social media also includes appropriately sharing factual and useful information.

Long term relationships are the mode of long term happiness (93, 98). Telephone and social media can be used to talk and asked about current situation andhealth, providing mental support, guiding people for proper at remote for proper hygienic life style, proving healthcare tips and advice or service by doctors, and many more. Consequently,it will create the opportunity to reconnect every single bond and stretch them with positive signature and elasticity in life. So citizens should make to conscious resolve connect with one's relationship and friends through social media and phone and make them aware and support each other. On the other hand, one should not be addictedwith it which makes a dangerous health issues. Positive engagement in social media can involve initiating or participating in interesting debates on many social issues to come up with solutions. Constructive discussions can promote learning opportunities, social bonding and the challenge the place of unhelpful attitudes and narratives.

***Online community participation:*** One may use social media to find new and creative ways to do group activities online together. This may be exercise classes, sewing lessons, watching a film at the same time and then discussing it (Netflix Party), gardening or potting tips or classes, language lessons, study/tutoring sessions or classes, meditations, music groups or even just coffee mornings. People are only limited by their imagination, and it is amazing what they can do from the comfort of their home which enables them to connect with other people. One can also use the time on social media to research and promote worthwhile causes and raise awareness on important issues that may have been neglected due to the fast-paced life that have been accustomed to living style. Positively engaging in social media with topics that one feels passionate about can help to find the voice and increase self-confidence and feelings of self-worth.

***Food to needy people, animals and birds:*** Most animals are fed due to daily human activities. Many people who feed animals stopped under the scare of COVID-19. Thousands of stray cats and dogs depend on food and waste from markets, restaurants and grocery shops. However, COVID-19locks people under social lockdown and public establishments have beenshuttled down; strays are having a hard time finding food. In the event of a lockdown, if they are not fed, many will die, creating another kind of risk in ecosystem. Therefore, every household can share a little fraction of their own food to stranded stray animals as well to birds. Same principles are also must be applied for the daily wages. In many counties, serious threats have been arrived for daily wages, although government is taking steps, being considered as insufficient. Therefore, humanity is challenged to provide them food security and it

can be easily solved with individual effort. In the midst of the darkness spread by the corona pandemic, the human being must continuously progress towards light and hope. They must continuously strive to take those who are most affected, the poor brothers and sisters, from disappointment to hope. It must end the darkness and uncertainty emanating from the crisis, by progressing towards light and certainty. Human being must defeat the deep darkness of the crisis, by spreading the glory of light in all four directions.

**Conclusion**

COVID-19 is the most dangerous pandemic the world had ever experienced. The causative agent is a mutated form of the previously existing Coronaviruses noticed in bat. No medicine or vaccines are available for the treatment or prevention of the disease although some of the drugs are repurposed; alternative medicines are needed to be screened using high throughput *in silico* tools to target the viral proteins. The response of such drugs may be environment and person specific as well (99-113). However, social distancing is considered as the only preventive measure to control or minimise the rate of infection and death. Regularly cleaning hands found to be most effective measure along with social distancing. Many developed countries with advanced health care system are failed to control the infection due to less strict social distancing maintained by their citizens. On the other hand, India being the world's second largest populous country where larger fraction of its citizen suffers from less hygienic life style, and with a weaker health care systems as compared to other developing and developed countries, has controlled the disease unexpectedly. This was achieved by observing a strict social lockdown that gives a hint to other nation to follow the social move strictly, because the prevailing medical care does not guarantee on cure of any infected person. On the other hand, it will provide the research, healthcare system to take care of the diseased people and hence huge load on the health care system will be reduced. It will also provide time to researchers to develop vaccines or specific medicines against for the treatment of COVID-19.

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# Glaucoma : A Review

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**Abstract**

Glaucoma is one of the most common ophthalmic conditions encountered in primary and secondary care**.** The glaucoma is a group of progressive optic neuropathies characterized by degeneration of retinal ganglion cells and resulting changes in the optic nerve head. It is usually associated with an increase in intraocular pressure (IOP) above the normal value—usually estimated at 21 mm Hg. Loss of ganglion cells is related to the level of intraocular pressure, but other factors may also play a role. Reduction of intraocular pressure is the only proven method to treat the disease. Although treatment is usually initiated with ocular hypotensive drops, laser trabeculoplasty and surgery may also be used to slow disease progression.

**Introduction**

Glaucoma is a leading cause of irreversible blindness throughout the world. In India glaucoma constitute 2% of total blindness (1). Moreover, the management of glaucoma has an enormous impact in our society in terms of patient's morbidity, loss of productivity, number of ophthalmic consultations and health costs, as these patients may have to continue the therapy for the whole life. However, new glaucoma medications have increased efficacy, reduced dosing frequency and improved side effect profiles; but there is need for comparing the cost of new glaucoma medication with the traditional one (2).

Glaucoma is an optic neuropathy in which the optic nerve is damaged with typical loss of nerve fibers and increasing cupping of the optic disc, leading to progressive, irreversible loss of vision. It is often, but not always, associated with increased pressure of the fluid in the eye. The nerve damage involves loss of retinal ganglion cells in a characteristic pattern. There are many different sub-types of glaucoma but they can all be considered a type of optic neuropathy. Raised intraocular pressure (IOP) is a significant risk factor for developing glaucoma (3).

Untreated glaucoma leads to permanent damage of the optic nerve and resultant visual field loss, which can progress to blindness. Glaucoma has been nicknamed the "sneak robber of sight" because the loss of vision normally occurs gradually over a long period of time and is often only recognized when the disease is quite advanced. Once lost, this damaged visual field cannot be recovered. Worldwide, glaucoma is the second leading cause of blindness and affects approx 66 million people in the world. In some countries, e.g. United States of America were approximately 100000 people are totally blind and approx 300000 are blind in one eye from glaucoma, it is the leading cause of blindness. Glaucoma affects 1 in 200 people aged fifty and younger, and 1 in 10 over the age of eighty (4, 5 & 6).

Glaucoma is one of the most common ophthalmic conditions encountered in primary and secondary care. The World Health Organization estimated that in 2010 glaucoma accounted for 2% of visual impairment and 8% of global blindness. Disability adjusted life years

attributable to glaucoma more than doubled between 1990 and 2010 due to the worldwide increase in the number of older people. Glaucoma is the leading cause of irreversible blindness in the world (7, 8).

Glaucoma was probably recognized as a disease entity in the 17 th Century where the term was derived from the Greek term glauk"oma meaning cataract or opacity of the lens implying the lack of understanding of this disease process. Today we understand that glaucoma is a group of diseases with common end point characteristics affecting the optic nerve. It is defined as an optic neuropathy characterized by specific structural findings in the optic disk (increased vertical cup disk ratio (VCDR). In the past, raised intraocular pressure (IOP) was used as a defining characteristic for glaucoma; but now IOP is considered as just an important risk factor for glaucoma (9).

However, surveys show that 20-52% of patients with glaucoma have IOP within the normal range. Primary open angle glaucoma is the most common type of glaucoma, accounting for over 70% of cases. It is an IOP related optic neuropathy that gives rise to characteristic optic disc changes and visual field loss. In its early stages it affects peripheral visual field only, but as it advances it results in loss of visual acuity and can cause blindness. Some patients with statistically normal IOP develop the characteristic changes associated with open angle glaucoma and are said to have low tension or normal pressure glaucoma (3, 10).

**Epidemiology**

It is estimated that there are more than 60 million cases of glaucoma worldwide and it will increase to 80 million by 2020 (11, 12) .The estimated prevalence of glaucoma is 2.65% in people above 40 years of age. Globally, primary open-angle glaucoma (POAG) is more prevalent than primary angle closure glaucoma (PACG) and responsible for around three fourth of all glaucoma cases. Overall glaucoma is the second major cause of blindness after cataract. It is the most common cause of irreversible blindness globally. It is estimated that more than 3 million people are blind due to glaucoma (13).

In India, the estimated number of cases of glaucoma is 12 million, around one fifth of the global burden. In the Indian population an equal proportion of open-angle and closed-angle glaucoma is seen (10,11 & 13).

**Etiology** (14, 15, 16 & 17) : The following are groups at higher risk for developing glaucoma.

**1. African Americans:** After cataracts, glaucoma is the leading cause of blindness among African Americans and people of African descent. Glaucoma is six to eight times more common in African Americans than in Caucasians.

**2. People Over 60 :** Glaucoma is much more common among older people. You are six times more likely to get glaucoma if you are over 60 years old.

**3. Family Members with Glaucoma :** The most common type of glaucoma, primary open-angle glaucoma, is hereditary. If members of your immediate family have glaucoma, you are at a much higher risk than the rest of the population. Family history increases risk of glaucoma four to nine times.

**4. Hispanics in Older Age Groups :** Recent studies indicate that the risk for Hispanic populations is greater than those of predominantly European ancestry, and that the risk increases among Hispanics over age 60.

**5. Asians :** People of Asian descent appear to be at increased risk for angle-closure glaucoma. Angle-closure glaucoma accounts for less than 10% of all diagnosed cases of glaucoma. People of Japanese descent are at higher risk for normal-tension glaucoma.

**6. Steroid Users :** Some evidence links steroid use to glaucoma. A 1997 study reported in the Journal of American Medical Association demonstrated a 40% increase in the incidence of ocular hypertension and open-angle glaucoma in adults who require approximately 14 to 35 puffs of steroid inhaler to control asthma. This is a very

**Table: 1** Types of Glaucoma and its causes

| GLAUCOMA TYPE | ETIOLOGY (16) |
|---|---|
| PRIMARY OPEN ANGLE GLAUCOMA | Drainage channels are partially blockedThis causes the fluid to drain out of the eye too slowly |
| PRIMARY CLOSURE ANGLE GLAUCOMA | Complete blockade of drainage channelThis causes increase in pressure |
| DEVELOPMENTAL GLAUCOMA | Congenital<br>glaucomaInfantile glaucomaJuvenile glaucomaIt could develop as the result of other conditions (secondary glaucoma) |
| PIGMENTARY GLAUCOMA | Pigment granules from your iris build up in the drainage channels (trabecular meshwork) |

high dose, only required in cases of severe asthma.

**7. Eye Injury :** Injury to the eye may cause secondary open-angle glaucoma. This type of glaucoma can occur immediately after the injury or years later.

Blunt injuries that "bruise" the eye (called blunt trauma) or injuries that penetrate the eye can damage the eye's drainage system, leading to traumatic glaucoma.

8. The most common cause is sports-related injuries such as baseball or boxing.

Signs and Symptoms (12, 18 & 19)

**OPEN-ANGLE GLAUCOMA**

- Most people have no symptoms
- Once vision loss occurs, the damage is already severe
- Slow loss of side (peripheral) vision (also called tunnel vision) as shown in fig: 1
- Advanced glaucoma can lead to blindness

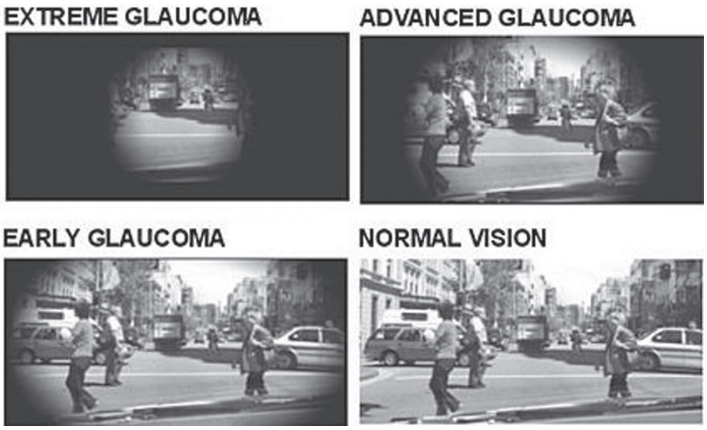


**Fig 1:** Tunnel vision observed by Glaucoma patient

**ANGLE-CLOSURE GLAUCOMA**

Symptoms may come and go at first, or steadily become worse (20, 21). Symptoms are as follows:

- Sudden, severe pain in one eye
- Decreased or cloudy vision, often called "steamy" vision
- Nausea and vomiting
- Rainbow-like halos around lights as shown in fig: 3
- Red eye as shown in fig :2
- Eye feels swollen

**CONGENITAL GLAUCOMA (21, 22)**

Symptoms are usually noticed when the child is a few months old.

- Light sensitivity (photophobia)
- Corneal opacification (hazy gray cornea)
- Enlarged eye and cornea
- Epiphora (overflow of tears)
- Vision loss

A cloudy cornea is the earliest and most common sign of childhood glaucoma. The healthy cornea is transparent. The loss of this transparency is caused by edema, or swelling of tissue from excess fluid. This occurs in the corneal epithelium (outermost layer of the cornea) and in the corneal stroma (middle layer of the corneal tissue). Careful inspection of the cornea may also reveal defects in its inner layer, which is further proof of a raised eye pressure (IOP).

In most cases of glaucoma affecting children under three years of age, the cornea and eye enlarges. Review of early photographs of your child may reveal the presence of glaucoma months before the diagnosis was actually made.

In addition to eye problems, secondary systemic (body) symptoms may occur. These secondary symptoms are especially common with acute glaucoma. Examples include irritability, loss of appetite, and vomiting. These symptoms may be misunderstood before the glaucoma is recognized. Young children with glaucoma are often unhappy, fussy, and poor eaters.

A slow chronic increase in eye pressure is probably not painful. In contrast, there is discomfort and pain when the eye pressure increases rapidly during an acute onset or with the rapid return of glaucoma following unsuccessful glaucoma surgery. Lowering high eye pressure relieves these painful symptoms quickly.

**SECONDARY GLAUCOMA (22)**

- Symptoms are usually related to the underlying problem causing the glaucoma
- Depending on the cause, symptoms may either be like open-angle glaucoma or angle-closure glaucoma

**Types of Glaucoma (13, 14)**

Glaucoma is actually a group of diseases. The most common type is hereditary.

- Primary Open-Angle Glaucoma
- Angle-Closure Glaucoma
- Normal-Tension Glaucoma
- Other Types of Glaucoma

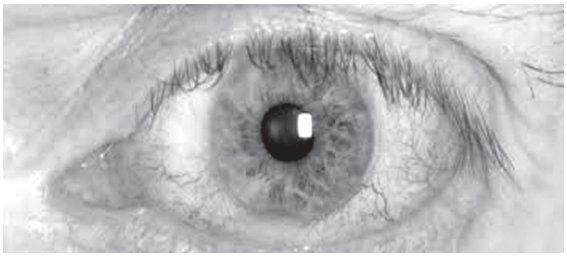

**Fig: 2** Red eye in Glaucoma patient

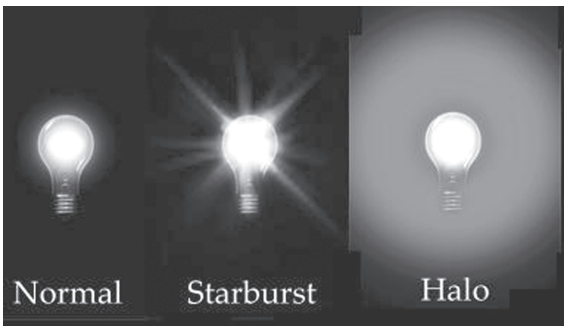


**Fig: 3** Rainbow like halos around lights

The two main types are open-angle and angle-closure. These are marked by an increase of intraocular pressure (IOP), or pressure inside the eye.

**Open Angle Glaucoma (23)**

Open-angle glaucoma, the most common form of glaucoma, accounting for at least 90% of all glaucoma cases:

- Is caused by the slow clogging of the drainage canals, resulting in increased eye pressure.
- Has a wide and open angle between the iris and cornea.
- Develops slowly and is a lifelong condition.
- Has symptoms and damage that are not noticed.

"Open-angle" means that the angle where the iris meets the cornea is as wide and open as it should be as shown in fig: 4. Open-angle glaucoma is also called primary or chronic glaucoma. It is the most common type of glaucoma, affecting about three million Americans (24).

**Angle-Closure Glaucoma (25, 26)**

Angle-closure glaucoma, a less common form of glaucoma:

- Is caused by blocked drainage canals, resulting in a sudden rise in intraocular pressure
- Has a closed or narrow angle between the iris and cornea
- Develops very quickly
- Has symptoms and damage that are usually very noticeable
- Demands immediate medical attention.

It is also called acute glaucoma or narrow-angle glaucoma. Unlike open-angle glaucoma, angle-closure glaucoma is a result of the angle between the iris and cornea closing as shown in fig: 5.

**Normal-Tension Glaucoma (NTG) (27)**

Also called low-tension or normal-pressure glaucoma, in normal-tension glaucoma the optic nerve is damaged even though the pressure in the eye is not very high. Doctors do not know why some people's optic nerves are damaged even though they have almost normal pressure levels.

Those at higher risk for this form of glaucoma are:

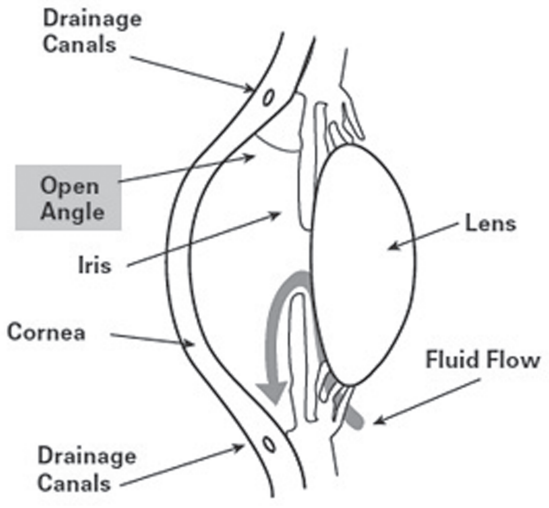


**Fig: 4** Drainage canal in Open Angle Glaucoma

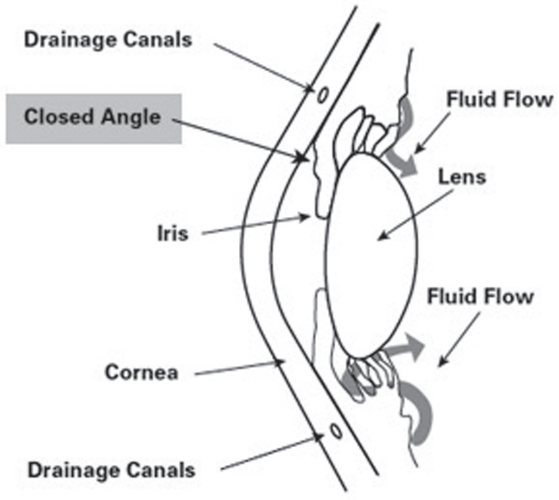


**Fig: 5** Drainage canal in Angle Closure Glaucoma

People with a family history of normal-tension glaucoma

People of Japanese ancestry (28)

People with a history of systemic heart disease such as irregular heart rhythm (29).

Normal-tension glaucoma (NTG), also known as low tension or normal pressure glaucoma, is a form of glaucoma in which damage occurs to the optic nerve without eye pressure exceeding the normal range. In general, a "normal" pressure range is between 12-22 mm Hg.

Childhood glaucoma — also referred to as congenital glaucoma, pediatric, or infantile glaucoma — occurs in babies and young children. It is usually diagnosed within the first year of life. This is a rare condition that may be inherited, caused by incorrect development of the eye's drainage system before birth. This leads to increased intraocular pressure, which in turn damages the optic nerve.

**Other Types of Glaucoma (30)**

Variants of open-angle and angle-closure glaucoma include:
Secondary Glaucoma
Pigmentary Glaucoma
Pseudoexfoliative Glaucoma
Traumatic Glaucoma
Neovascular Glaucoma
Irido Corneal Endothelial Syndrome (ICE)

Secondary glaucoma refers to any form of glaucoma in which there is an identifiable cause of increased eye pressure, resulting in optic nerve damage and vision loss.

As with primary glaucoma, secondary glaucoma can be of the open-angle or angle-closure type and it can occur in one or both eyes. Secondary glaucoma may be caused by an eye injury, inflammation, certain drugs such as steroids and advanced cases of cataract or diabetes. The type of treatment will depend on the underlying cause, but usually includes medications, laser surgery, or conventional surgery.

Glaucoma develops in some patients with a condition called exfoliation syndrome. Also known as pseudoexfoliation, it is caused by the abnormal accumulation of protein in the drainage system and other structures of the eye. This is a type of open-angle glaucoma with unique characteristics and physical findings. It is more common in certain racial groups including people from Russia, the Nordic countries, Greeks, Mediterranean populations, Indians, and others.

A gene abnormality has recently been associated with this particular condition. As a group, patients with exfoliative glaucoma show higher pressures and faster disease progression than patients with classic primary open-angle glaucoma. The underlying cause is likely due to the abnormal protein and associated pigment blocking the outflow structures in the eye.

Patients with exfoliative glaucoma often have more episodes of high pressure, more fluctuations and higher peak pressures than patients with other types of glaucoma. Generally, this kind of glaucoma is more difficult to control with medical therapy. Patients with exfoliative glaucoma often require a more aggressive stepwise therapy and more often need laser, or incisional surgery. Often more frequent visits to their eye doctor are necessary to monitor for disease progression. Exfoliative glaucoma patients seem to respond well to treatment by laser trabeculoplasty, possibly because of the more pigmented meshwork and a higher concentration of enzymes in the meshwork, termed matrix metalloproteinases, that are activated by laser trabeculoplasty. Patients with this disorder respond well to most types of glaucoma surgery. However, whether or not they respond well to trabecular stent devices or the new generation of tubes to the suprachoroidal space remains to be seen.

Another reason to know whether or not exfoliation is present is that these patients sometimes have increased difficulty with cataract surgery. The abnormal protein seen in this condition settles on and weakens the lens zonules which are suspensory fibers that hold the lens in

place. In most patients, the surgical technique can be modified to obtain a good outcome.

**Neovascular Glaucoma** is caused by the abnormal formation of new blood vessels on the and over the eye's drainage channels. Neovascular glaucoma is always associated with other abnormalities, most often diabetes. It never occurs on its own. The new blood vessels block the eye's fluid from exiting through the trabecular meshwork (the eye's drainage canals), causing an increase in eye pressure.

**Pigmentary Glaucoma** occurs when the pigment granules that are in the back of the iris (the colored part of the eye) break into the clear fluid produced inside the eye. These tiny pigment granules flow toward the drainage canals in the eye and slowly clog them, causing eye pressure to rise.

**Traumatic Glaucoma** — Injury to the eye may cause traumatic glaucoma. This form of open-angle glaucoma can occur immediately after the injury or develop years later. It can be caused by blunt injuries that bruise the eye (called blunt trauma) or by injuries that penetrate the eye.

**Uveitic Glaucoma** — Uveitis is swelling and inflammation of the uvea, the middle layer of the eye. The uvea provides most of the blood supply to the retina. Increased eye pressure in uveitis can result from the inflammatory process itself or the medication (steroids) used to treat it**.**

**Pathophysiology :** Several large studies have shown that eye pressure is a major risk factor for optic nerve . In the front of the eye is a space called the anterior chamber. A clear fluid flows continuously in and out of the chamber and nourishes nearby tissues. The fluid leaves the chamber at the open angle where the cornea and iris meet. When the fluid reaches the angle, it flows through a spongy meshwork, like a drain, and leaves the eye (31).

In open-angle glaucoma, even though the drainage angle is "open," the fluid passes too slowly through the meshwork drain. Since the fluid builds up, the pressure inside the eye rises to a level that may damage the optic nerve as shown in fig: 6. When the optic nerve is damaged from increased pressure, open-angle glaucoma—and vision loss—may result. That's why controlling pressure inside the eye is important.

Another risk factor for optic nerve damage relates to blood pressure. Thus, it is important to also make sure that your blood pressure is at a proper level for your body by working with your medical doctor. Progress made in the past few years in medical research has allowed a new approach to the pathophysiology of glaucoma, by studying the pathologic process on a tissue, cellular, molecular and genetic level (32). Following factors are responsible for causing glaucoma

**1. Role of vascular factors :** Some studies have shown a link between ocular perfusion pressure and glaucoma, as it is known that glaucoma can progress despite low intraocular pressure. Progression in case of normal tension glaucoma has been associated with deficiencies in the mechanism of regulation of ocular circulation. Interestingly, reduction of blood flow was also observed in the nail-fold capillaries of fingers in glaucoma patients suggesting that the reduction of blood flow is not due to increased IOP or an epiphenomenon of glaucoma, but a global vascular dysregulation is involved in POAG especially in NTG cases.

Researchers have proved that the long term changes in retinal circulation can lead to glaucoma-like aspects of the optic disc, independent of intraocular pressure (33).

**2. Glaucoma and Neurodegenerative Disorders :** Recent findings, no longer consider glaucoma as an autonomous dysfunction, affecting a single population of cells— the retinal ganglion cell fibers. More and more data suggest that glaucoma should be integrated in the category of neurodenerative diseases (24).

**3. Oxidative stress of the Retinal ganglion cell layer :** It has been proved that the death of retinal

ganglion cells in glaucoma occurs through apoptosis. It is thought that increased oxidative stress, due to high levels of free radicals, can induce apoptosis of retinal ganglion cells and is thus involved in the pathogenesis of glaucomatous optic neuropathy (25, 26).

**4. Role of serotonin :** Serotonin is a neurotransmitter synthesized in neurons and deposited in intracellular vesicles. Serotonin is present in high amounts in the iris-cilliary body complex and seems to play a part in regulating the flow of the aqueous humor.

Seven types of serotoninergic receptors have been identified (from 5-HT1 to 5-HT7). Stimulation of 5-HT7 leads to an increase of intraocular pressure, while stimulation of 5-HT1 leads to decrease in intraocular pressure (27).

**Diagnosis**

**1. Visual field testing :** During a routine eye exam, some eye doctors may want to determine through visual field testing the full horizontal and vertical range of what you are able to see peripherally as shown in fig: 7. This range is commonly referred to as "side vision." Visual field tests assess the potential presence of blind spots (scotomas), which could indicate eye diseases (34).

**2. Dilated eye exam :** In this exam, drops are placed in your eyes to widen, or dilate, the pupils. Your eye care professional uses a special magnifying lens to examine your retina and optic nerve for signs of damage and other eye problems as shown in fig: 8. After the exam, your close-up vision may remain blurred for several hours (35).

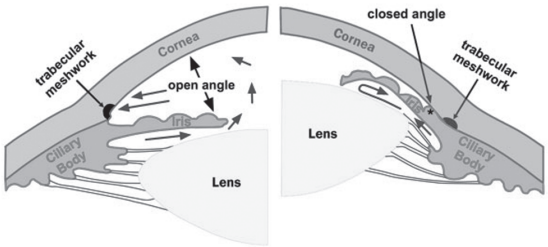


**Fig: 6** Fluid pathway in Glaucoma

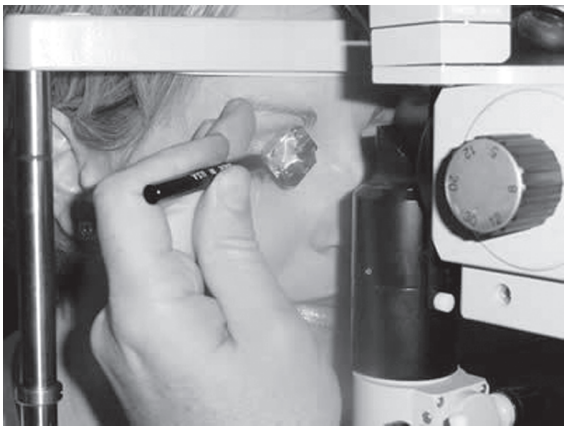

**Fig: 8** Examination of eye during dilated eye exam test

**Fig: 7** Visual field testing

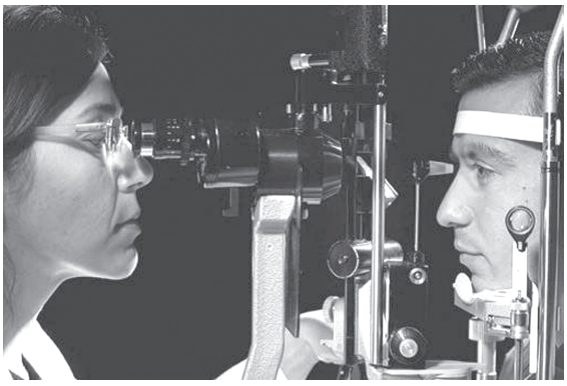

**Fig: 9** Glaucoma testing through tonometry

**3. Tonometry :** It is the measurement of pressure inside the eye by using an instrument (right) called a tonometer as shown in fig: 9. Numbing drops may be applied to your eye for this test (36).

**4. Pachymetry :** It is the measurement of the thickness of your cornea. Your eye care professional applies a numbing drop to your eye and uses an ultrasonic wave instrument to measure the thickness of your cornea (36).

**5. Gonioscopy** : It is performed to make sure the aqueous humor (or "aqueous") can drain freely from the eye. In gonioscopy, special lenses are used with a biomicroscope to enable your eye doctor to see the structure inside the eye (called the drainage angle) that controls the outflow of aqueous and thereby affects intraocular pressure. Ultrasound biomicroscopy is another technique that may be used to evaluate the drainage angle (37).

**Treatment :** Treatment can involve glaucoma surgery, lasers or medication, depending on the severity. Eye drops with medication aimed at lowering IOP usually are tried first to control glaucoma. Because glaucoma often is painless, people may become careless about strict use of eye drops that can control eye pressure and help prevent permanent eye damage.

**Medicines**

Medicines, in the form of eye drops or pills, are the most common early treatment for glaucoma. Taken regularly, these eye drops lower eye pressure. Some medicines cause the eye to make less fluid. Others lower pressure by helping fluid drain from the eye (38).

**Surgery**

**Laser trabeculoplasty**

Laser trabeculoplasty helps fluid drain out of the eye.

Before the surgery, numbing drops are applied to your eye. As you sit facing the laser machine, your doctor holds a special lens to your

**Table: 2** Drugs for the treatment of Open Angle Glaucoma (39)

| | |
|---|---|
| OPEN ANGLE GLAUCOMA | |
| β ADRENERGIC BLOCKERS | TIMOLOL |
| | BETAXOLOL |
| | LEVOBUNOLOL |
| β ADRENERGIC AGONISTS | DIPIVEFRINE |
| | APRACLONIDINE |
| | BRIMONIDINE |
| PROSTAGLANDIN | LATANOPROST |
| ANALOGUES | TRAVOPROST |
| | BIMATROPOST |
| CARBONIC ANHYDRASE | ACETAZOLAMI |
| INHIBITORSMIOTICS | |
| | DEDORZOLAMIDE |

**Table: 3** Drugs for the treatment of Angle Closure Glaucoma (40)

| |
|---|
| ANGLE CLOSURE GLAUCOMA |
| HYPERTONIC MANNITOL |
| ACETAZOLAMIDE |
| MIOTICS and TOPICAL β BLOCKERS |

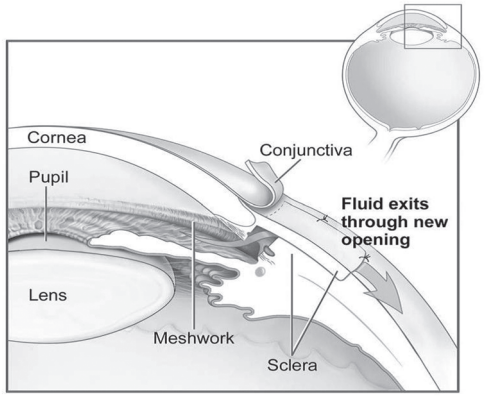


**Fig: 10** Conventional surgery makes new opening Sometimes after conventional surgery, your vision may not be as good as it was before conventional surgery. Conventional surgery can cause side effects, including cataract, problems with the cornea, inflammation, infection inside the eye, or low eye pressure problems (42).

eye. A high-intensity beam of light is aimed through the lens and reflected onto the meshwork inside your eye. You may see flashes of bright green or red light. The laser makes several evenly spaced burns that stretch the drainage holes in the meshwork. This allows the fluid to drain better (41).

**Conventional surgery**

Conventional surgery makes a new opening for the fluid to leave the eye. Conventional surgery, called trabeculectomy, is performed in an operating room. Before the surgery, you are given medicine to help you relax. The doctor makes small injections around the eye to numb it. A small piece of tissue is removed to create a new channel for the fluid to drain from the eye as shown in fig.10. This fluid will drain between the eye tissue layers and create a blister-like "filtration bleb." Conventional surgery is about 60 to 80 percent effective at lowering eye pressure. If the new drainage opening narrows, a second operation may be needed.

**Prevention**

Researchers in the U.K. found that higher levels of physical exercise appear to provide a long-term benefit of reducing the incidence of low ocular perfusion pressure (OPP), an important risk factor for glaucoma. OPP is a mathematical value that is calculated using a person's intraocular pressure and his or her blood pressure. The results showed that study participants who engaged in moderate physical exercise approximately 15 years prior to the study had a 25 percent reduced risk of low OPP that could lead to glaucoma.

"It appears that OPP is largely determined by cardiovascular fitness," said study author Paul J. Foster, MD, PhD, of the University College London Institute of Ophthalmology.

"We cannot comment on the cause, but there is certainly an association between a sedentary lifestyle and factors which increase glaucoma risk."

Maintaining an active lifestyle appears to be an effective way for people to reduce their risk of glaucoma and many other serious health problems (30).

**Discussion and Conclusion**

Glaucoma is a common eye disease that is usually associated with an elevated intraocular pressure. Treatment options for patients with glaucoma include medications, laser therapy, and incisional surgery. The risks and benefits of each type of treatment must be carefully considered to maximize the treatment's benefits while minimizing adverse effects

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# Review on taxonomical and pharmacological status of *Dolichos lablab*

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**Abstract**

*Dolichos lablab* is a fancy plant in the U.S., an accepted food in Asia, Africa, and Central & S. America. *Lablab* seeds are no more than 1/2 inch in length at maximum & is coloured white to brown, red and black. It is a climbing plant with broad and large leaves. It condones the pH range of 5 to 7.5 and flourishes in a various type of soils like deep sands to heavy black clays. This study concentrates on the origin, chemical composition, distribution, pharmacology and taxonomic status of the aforementioned plant. The pharmacology study showed that *D. lablab* possessed Antimicrobial, Antidiabetic, Analgesic, Antiinflammatory, Antioxidant, , hypolipidemic, Cytotoxic, Insecticidal effects.

**Keywords :** *Dolichos lablab,* Seed, Flower, Linoleic acid , Plant

**Origin and Distribution :**

Dolichos lablab or Lablab purpureus L. (Sweet) is an inhabitant of India (Murphy and Colucci, 1999) or Asia (South-East). It is likely originated from Asia & had been planted for long enough time. India grows a wild variety of the Lablab. D.lablab was possibly taken to tropical part of Africa and from there it may be spread across Caribbean, Malaysia, Sudan, Indonesia, Papua New Guinea, Philippines, Mainland China, Egypt, East and W. Africa, C. and S. America, etc.

Beans of D. Lablab is a very old crop in India and was likely to be found from before 3500 BC as mentioned by Fuller (2003). However, Maass et al. (2005) had no evidence as such through AFLP studies but to believe that the crop grew in eastern and/or southern Africa. As a matter of fact, the Indian counterpart of Lablab was genetically found to be a blend of the wild and the cultivated forms through the molecular marker studies. And that these forms failed to exist among the African collections led Maass et al. (2003) to hypothesize that the pattern of distribution of *D.lablab* was from Africa to Asia. This was additional thoroughbred by Maass et al. (2007) through studies concerning changes in seed characteristics between cultivated and wild variety. Still there's a faculty of thought, that believes within the twin centers of origin–Africa and Asia. Hoshikawa (1981) according that it absolutely was introduce in Japan from China in 1654 wherever it's known as “Fujimame” & young pods are used as vegetables.

In 1754, Linneus represent the species under D. lablab whereas in 1763 Adanson named *Dolichos L.* as Lablab. Firstly these species combined in L. adans was Lablab niger of Medikus (1787), based on *Dolichos lablab* L. A new epithet was required as Lablab would be tantonymous. Since L. niger is synonymous with *Dolichos lablab,* the 2 genera are homotypic by lectotypification.

Roxburgh (1832) described seven varieties of Dolichos in Flora India, out of 7, 5 varieties were used as food and the rest 2 are wild. The former 5 was again divided into 2 categories: (a) D. lablab var. typicus and (b) *D. lablab* var. lignosus (Barker, 1911). Moreover, cultivated varieties was divided as short-day with a photoperiod of 10 –12 hours and thus the others comparatively unaffected by day length (Rivals, 1953).

In 1965, Verdcourt anticipated the refusal of *D. Lablab* & in its place conservation of *Dolichos* Lam. with D. uniflorus Lam. as a type, because

*D. lablab* with lectotype *Dolichos lablab* L. would limit the generic name Dolichos to *Dolichos lablab.* Though, the team for Spermatophyta (1968) refused the proposal for three major reasons: (1) Proposed species is not one of those initially integrated by Linnaeus; (2) name Lablab Adanson would need conserving; (3) Scientist not in agreement with the segregation of Lablab & other generic splits could not use the name Dolichos L. for the combined genus.

**Alternate Names**

Alternate Common Names: Bonavist Bean, Field Bean, Egyptian Bean, Pendal Bean, Hyacinth Bean, Pole Bean (Gowda, 2013; Tropical Forages).

**Chemical Composition:**

a) Crude protein: Mean of crude protein present in lablab herbage was ranges from 17% to 22% on a dry matter basis. crude protein in Leaf ranges from 14.3% - 38.5%, whereas protein in stem content varied from 7%- 20.1%. As the Lablab crop matures, protein contents reduced (Milford and Minson 1968). Though, Schaaffhausen (1963b) marked that tannins are absent in lablab leaves, thus provides a quickly fermentable protein source with less by pass protein potential. Digestibility of the crude protein present in lablab forage has been compared with sheep (Jakhmola and Pathak 1981) & cattle (Hendricksen et al 1981), with coefficients range of 54.5 to 76.1%, depending upon the content of crude protein. Further study is necessary to get a proper result of the protein profile of L. purpureus and its various plant fractions.

b) Anti-nutritional characteristics: A major limitation to the utilization of legumes in diets of animal showed the presence of anti-nutritional factors. Schaaffhausen (1963b) reported that the lablab leaves is absent of tannins, showing that it is an truthful feed for monogastric animals. The seeds contain anti-nutritional factors like phytate, tannins and enzyme inhibitors. Compound activity may well be reduced by process ways like removing the reproductive structure, soaking and cookery (Lambourne and Wood 1985; Deka and Sarkar 1990).

c) Fibre Content: The mean crude fibre present in entire plant is 27.8% with an average of NDF-43 %, ADF-38.6%, and ADL-7.1% (Murphy and Colucci, 1999).

**Taxonomic Status :**

| | | |
|---|---|---|
| Order | : | Fabales |
| Family | : | Fabaceae |
| Subfamily | : | Faboideae |
| Species | : | Lablab purpureus |
| Tribe | : | Phaseoleae |
| Sub-tribe | : | Faboideae |
| Genus | : | Lablab |

**Growth Habit :** Plant is a perennial herb, frequently developed as an annual reaches 5-29 feet (Valenzuela and Smith, 2002) but likely bushy, semi-erect & prostrate. Perhaps such variation did not shown in form and habit. Well developed tap roots with many laterals and adventitious roots are also developed well.

2 types of Lablab Purseglove (1968) in India has been recognized and are often well thought-out as distinct species. It is:

*Dolichos lablab* var. typicus Prain: It is known as Lablab bean, Hyacinth bean, Bonavist bean, Indian butter bean (Hindi-Sem; Tamil-Avarai; Gujarathi-Val; Marathi-Pavta; Bengali-Shim; Malayalam- Avara Telugu-Chikkudu; Kannada-Chapparadavare;.)

*D.lablab* cultivated annually, distributed all through the temperate and tropical regions of America, Africa and Asia. In India known as a garden crop. They differ in flowers color, size, shape of pods and size of seeds, weight of seeds and color of seeds. In temperate regions, seed with purple color flowers is an ornamental plant. The pods are white to green or purple-margined whereas seeds are brownish, purple, yellow, white or black.

*Dolichos lablab* var. lignosus Prain: Commonly known as Field Bean, Australian pea. (Hindi-Ballar; Tamil – Mochai, Gujarati - Val; Telugu - Anumulu; Malayalam – Mochakotta, Kannada - Avare). The herb is bushy, semi-erect and cultivated annually. It shows little or no affinity to climb.

Leaflets are trifoliate and are small than those of var. typicus. Flowers borne on a straight stalk, more likely a foot high on which they unwrap in succession. Pods are oblong, broad, flat, firm-walled and fibrous with 4-6 long axis seeds at right angles. Seeds are likely to be round brown, white or black.

**Stem :** Stems are Cylindrical and twining at a height of 6m , glabrous or hairy, often 2-3 m, but usually to 10 m in length. Other forms are likely to be dwarf and bushy.

**Leaves :** Trifoliate and alternate leaves , with ovate leaflets, measuring 5- 14 cm x 4-14 cm, usually hairy. On the other hand leaflets are ovate, very broad, the lateral ones are lopsided, 7.5 -15 cm in length and nearly as broad, rather abruptly acuminate.

**Inflorescence :** The complete flower head is usually stiff and has a auxiliary raceme with many flowers. The peduncle is 4 -8.5 x 13.5 -23 cm and compressed mostly and glabrescent , the rachis is 2.5cm -6cm to 13.0cm -24.5 cm while the flowers, on rachis 1-5 together form tubercles, per flower one bracts is present, deciduous, elliptic to ovate, 5 mm in length having veins, sparsely pubescent; pedicels small and square, with 2 bracteoles attached to the calyx at the base.

**Flowers :** Flowers color is generally pinkish, white, reddish or purple and are found in group of 4 to 5. Every flower contains 2 large basal bracts, uniform anthers, upper 2 sepals connate are white to purple to pink or, 2-4 at every node in an elongating raceme upto 2.5 cm in length. Stamens diadelphous (9+1), stamen is long, free, flattening and genicorlate close to the base. Anther ellipsoid, basified irregularly, minutely denticulate and yellow. sessile Ovary with 10 mm in length, finely pubescent. Four ovulate are lengthy beaked with inner ovarial wall speckled having brown dots, style abruptly upturned, 8 mm in length, laterally compressed, apical part thinly pubescent, persistent on pod. Stigma glandular and capitate.

**Pods :** Regardless of the texture, shape or colour, the pods are roughly 5 - 20 cm elongated and 1-5 cm broad. They could be deeply curved ventrally, and shaped almost straight or like a crescent. The thick fleshy pods are found in the cultivars used as vegetables and lack any fibre. Some pods are septate meaning one seed in one compartment within the pod while others have a inflated appearance and are known as non-septate. In general, there are 3-6 seeds in each pod and usually measures as 1.25 cm in length at max.The hilum is always prominent, white and has an oblong shape covering 1/3 of the seed generally. The seeds vary in color from white, cream or red to black and brown and also might be flat, oval r round.

**Seeds :** The seeds usually vary in size between 5-10 cm in length (Cook et al., 2005; Valenzuela and Smith, 2002) and are colored white, red, brown, black or speckled. The shape of the seeds are in general rounded or oval with a prominent white hilum which is approximately 10 mm in length,. 100 seeds on an average weighs 25 - 40 g. Germination is epigeal.

**Pharmacology :**

**Antibacterial Activity :** Chadalavada et al. (2015) reported antibacterial effect of methanol, ethanol, aqueous extracts of *Dolichos lablab* and four strains of bacterium (Escherichia coli, P. aeruginosa, B.subtilis & Staphylococcus) victimization agar well diffusion methodology. Among the 3 extracts, methanolic extract showed terribly potential bactericide activity with maximum inhibition zone against 2 Gram (-) bacterium E. coli, P. aureginosa, with inhibition zone of 8-11mm.

Priya and Jenifer (2014) evaluated Methanol extract of Leaf and Flower for antibacterial activity against S. aureus by using Agar well diffusion method. The phytochemicals there in the plant are to be extracted for antibacterial activity.

**Antifungal Activity :** Chadalavada et al. (2015) reported antifungal effect of methanolic, Ethanolic & Aqueous extracts of *Dolichos lablab* and 3 strains of fungi (T. rubrum, T. mentagrophytes, C. albicans.) by using well diffusion methodology. Among the 3 extracts, methanol extract showed maximum antifungal activity against T. mentagrophytes (30 mm) at a range 15-19mm.

**Anti-inflamatory Activity :** Anti-inflammatory activity of lablab was reported by Habib et al., 2012. The activity was performed as described21 by 1ml of extract solution transfer from the mother test tube to the respective marked test tube.0.03mg (2 or 3 finepart) of trypsin is added in each test tube. Then 100µl of 25mM tris HCl (pH-7.4) is added. 1ml of 0.8% (weight/volume) in case of solution is mixed. Wait for 20 minutes. 2ml of HclO4 (70%) is also added to terminate the reaction. Cloudy suspension is centrifuged at 3000 rpm for 30 minutes. At last the absorbance of supernatant was taken at 280nm against buffer as blank. It shows that with concentration the absorbance is decreased it means %inhibition is increased with concentration and there is a significant anti-inflammatory effect. It can be inferred from the figure 1 that there is a significant anti- inflammatory activity compared with the results found by Kumarappan et al. Though, study on animal and more studies is required for its activity and moreover studies required to elucidate its mechanism of anti– inflammatory action.

**Antioxdent Activity :** A molecule that ends any chain reaction by removing free radical intermediates while prohibiting any other sort of oxidation reactions, is called an oxidant. Linoleic acid–emulsion systems used to study this property of the Dolichos lablab and the e preoxidation of Linoleic acid was investigated. At 250µg in the final reaction mixture, *Dolichos lablab* inhibits 98.58% preoxidation of L. acid after incubated for 54hrs at 1 mg conc. in the final solution. Tsuda et al. (1993b) also studied that the extract of horse gram in Methanol inhibit lipid preoxidation activity in the L. acid. The current investigation also established similar observations. Generally, antioxidants for example phenolic compounds present in the seed coat play an significant role in protecting from oxidative damage. A different vary of high and low Mol. weight plant polyphenolics showed antioxidant activity is studied and planned to protect from lipid protection (Hagerman et al.1998). Jiratanan and Liu (2004) studied that thermal processing of B.vulgaris and P.vulgaris had no negative effect against antioxidant activity.

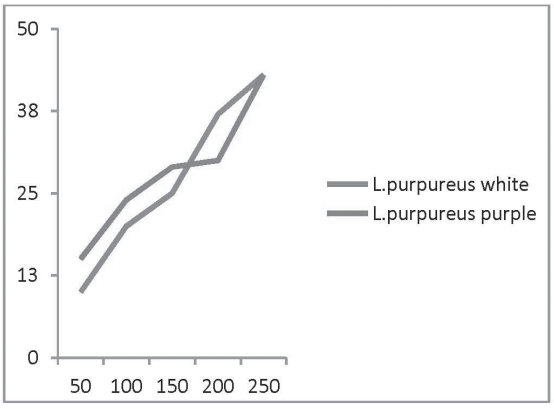


**FIGURE 1:** ANTI-INFLAMMATORY TEST OF METHANOL EXTRACT OF LABLAB PURPUREUS L.

**Antidiabetic Activity :** Kante and Reddy (2012) Treated diabetic rats with MEDL dose dependely ($P < .001$) decrease the level of blood glucose, cholesterol as a whole, triglycerides, SGPT, and SGOT levels as compared with diabetic rats which were untreated and kept in STZ induced diabetic model. MEDL 400mg/kg b.wt was found to possess more effective Antidiabetic activity compare to 200mg/kg body wt.

**Anti-Hyperglycemic Activity :** Tests of Glucose tolerance are performed orally to determine the antihyperglycemic trait of the plant under research. The baseline glucose level in the blood of fasted mice increased from 3.93 ± 0.13 mmol/L to 5.98 ± 0.20 mmol/L post the study. The glucose loaded mice showed evidence of significantly reduced glucose level on charging MEPL at conc. of 50 mg, 100mg , 200mg and 400mg per kg body weight. Percentage of glucose level reduction on administering the aforementioned doses were 6.4, 39.1, 40.1, and 54.8 respectively. Comparative study says any standard antihyperglycemic drug reduces blood glucose level by 53.8% in glucose induced mice when given at a conc. of 10mg/kg body weight (Rahmatullah et al.. 2015).

**Antinociceptive Activity:** This activity is established through abdominal writhing test.

Interpertoneal administration of acetic acid that leads to abdominal constrictions were highly reduced in a dose-dependant method. The percentage of number of constrictions reduction on administering the doses at 50mg, 100mg, 200mg and 400mg/kg body weight, were 32.3, 45.2, 54.8, and 58.1 respectively. Comparative study says any standard antinociceptive drug like aspirin, reduces umber of abdominal constrictions by 48.4% & 61.3% in experimental animals when given at conc. of 200mg/kg and 400mg/kg b. wt..

**Antacid Activity:** Formulation of the Dolichos lablab bean extract in Polyherbal has anti effects for acidic behaviour (Wu et al., 2010).

**Gastroprotective Activity:** Dolichos lablab leaves indicated Gastroprotective effects against ulcers influenced by ethanol and aspirin (Tarin and Chichioco-Hernandez, 2011).

**Cytotoxic Effect:** A brine shrimp lethality test was conducted on the sweet white and purple to study cytotoxic effect of methanolic extracts of 2 bean pods of Bangladeshi L. purpureus. The study showed the LC50 value to be 960.06 µg/ml for the sweet purple variety whereas it was 66.5 µg/ml in case of the sweet white, thereby proving that the white variety was more potent than the other [Habib et al., 2012]. Crude extracts from L. purpureus leaves did also undergo Brine Shrimp Lethality Bioassay tests to understand the cytotoxic effect. The result was then compared against LC50 values of standard Vincristin sulphate measured to be the +ve control. Results against A. salina thus found proved major cytotoxicity, with LC 50 13.88µg/ml for n-hexane, 19.17µg/ml for Chloroform, and 17.97µg/ml for Ethyl acetate extracts. [Nasrin et al.,2012].

**Hypolipidemic Effect:** A study of the bean germinated in India was carried out on rats with high levels of fats (lipids), such as cholesterol, in the blood to see the hypercholesterolemic effect of Dolichos lablab. The diet when supplemented with the powder made from the soaked bean of the corresponding variety, reduced plasma cholesterol from 178mg/dl? to 72.5mg/dl in comparison to control i.e, 61.5?±?0.70. Cholesterol level of the liver was however still 3 times greater than the control. As per researchers, the Indian bean cotyledons when sprout through 24 hours counteracts the additional liver and plasma cholesterol effectively. They do so by means of their high fibre content in parallel to ascorbic acid level being raised to a vast extent.

**Insecticidal Effect:** The extract from the seed of the Indian wild variety of Lablab purpureus is a protein named Arcelins. This protein, as per anarthanan et al., 2012, had nsecticidal activity against Callosobruchus. Studies showed that 2% of Arcelins incorporated in regular diet can reduce the development of Oryzaephilus surinamensis and Rhyzopertha dominica. A greater proportion of 5% of the same protein, however, led to the entire mortality of all larvae of R. dominica and O. surinamensis [Janarthanan et al., 2008].

**Conclusion:**

The effects of studies related to biology such as Antidiabetic, Antiinflammatory, Analgesic, Antioxidant, Cytotoxic, Hypolipidemic, Antimicrobial, Insecticidal were observed in Dolichos lablab. The extract from the beans of this plant can help n reduction of any pain or blood sugar lowering. A wide area exists where studies are yet to be done to fully recognise this specie. It is believed that seasonal variations or geographical factors could also contribute to the chemical components of the plant that are responsible for many of its activities and this can be a subject of greater interest. There is thus the need of educating people about the Dolichos lablab and also raise the awareness in pharmacologists and the researchers so that a better deed can be done in the the society through medical values. Writing reviews along with continuous research on the various aspects of the particular specie, thereby increasing the interest of various research communities is the only way to create medicinal progress with Dolichos lablab.

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# Angiotensin-converting enzyme gene polymorphism may be a risk factor for COVID-19 clinical outcome

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**Abstract**

Angiotensin-converting enzyme 1(ACE1) and ACE2 play a major role in regulation of blood pressure and electrolytic balance. They are known to express in epithelial cells of various tissues. SARS-CoV2 uses ACE2 as one of the receptors to enter into the host cells. Coronavirus infection-associated decrease in the expression of ACE2 is known to associate with vasoconstriction, hypertension and other cardiovascular problems. Patients who are on ACE1 inhibitors show increased ACE2 expression, which is known to protect the lung from acute lung injury. ACE1 and ACE2 polymorphisms might be associated with the infectivity, severity and recovery from the COVID-19. Association studies of ACE gene polymorphisms in affected population may suggest the clinical outcome of the COVID-19.

**Keywords :** Angiotensin-converting enzyme; polymorphism; COVID-19; spike protein

Contracting a disease is dependent on individual's genetic makeup in several cases. It is true in metabolic disorders and infectious diseases too. Even in case of viral infections, some people showed inherited resistance against HIV infection (Zimmerman et al. 1997). Among coronaviruses, SARS-CoV and MERS-CoV resulted in 10% of case-fatality rate during 2003 epidemic (Barnard et al. 2011) and up to 40% mortality rate in some regions during 2012 (Al Awaidy et al. 2019) respectively. This is due to, partly, extreme genetic variability of RNA viruses such as coronaviruses, increased transmission rate and recombination that occur in viruses when they are in reservoir animals (Barnard et al. 2011). The recent pandemic, COVID-19, accounts for 5103006 infected cases and 333401 deaths as on May 23, 2020 (https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200523-covid-19-sitrep-124.pdf? sfvrsn=9626d639_2). Though good supportive care was given and maintaining social distancing, the infective cases and death toll is being increased continuously. The SARS-CoV (Li et al. 2003) and COVID-19 (Zhou et al. 2020) were found to use angiotensin-converting enzyme 2 (ACE2) as one of the receptors to enter into host cell (Panati et al. 2020). In recent COVID-19 pandemic, older individuals and the individuals with cardiovascular diseases were observed to be at the high risk in China (Wu et al. 2020) and other places.

The renin–angiotensin system (RAS) is a key player in maintaining the homeostasis of blood pressure and electrolyte balance and is a marker for essential hypertension (Nicholls et al. 1998) and COVID-19 (Roncati et al. 2020). ACE1 gene is mapped to chromosome 17q23 and consisting of 25 exons and 26 introns. A 287 bp *Alu* repeat sequence inclusion plays altered levels of ACE1 and its activity. The insertion/deletion (I/D) polymorphism was found in intron 16 of ACE1 gene. The homozygous deletion genotype (DD) results in higher levels of ACE1 protein in plasma. Heterozygous condition shows moderate levels

and homozygous insertion genotype (II) results in low levels of ACE1 (Rigat et al. 1990). ACE1 converts angiotensin-I into angiotensin-II, in-turn results in vasoconstriction, hypertension and cardiac hypertrophy. ACE1 polymorphism (I/D) has been shown to be linked with coronary artery disease in Chinese Hans population also (Zhang et al. 2019). In systemic arterial hypertension, ACE1 (I/D) and ACE2 (G8790A) polymorphisms were found associated in Brazilian patients (Pinheiro et al. 2019). It indicates that the DD genotype of ACE1 might be associated with hypertension. Moreover, it was found that an association of DD genotype of ACE1 with hypoxemic condition in SARS-CoV infected cases (Itoyama et al. 2004). The other homologue of ACE1, ACE2, shows some sequence similarity with ACE (40% identity and 61% similarity) but functionally different, especially in substrate specificity. It is mapped to the chromosome Xp22 (Tipnis et al. 2000) and plays a protective role in cardiac function. ACE2 is known to convert angiotensin-II (DRVYIHPF) into angiotensin 1-7(DRVYIHP) that functions as vasodilator, which is opposite to ACE1 activity (Donoghue et al. 2000). Targeted disruption of ACE2 in mice resulted in a severe cardiac contractility defect, increased levels of angiotensin-II, over expression of hypoxia-induced genes in the heart (Crackower et al. 2002). ACE2 is known to be expressed by epithelial cells of the lung, intestine, kidney, and blood vessels. ACE2 also plays an important role in protecting from acute lung injury (ALI)/ acute respiratory distress syndrome (ARDS). *Ace2* gene knockout mice showed increased severity of lung injury compared to wild type in acid-aspiration-induced, endotoxin-induced and peritoneal sepsis-induced ARDS mice models (Imai et al. 2005). The mice deficient in *Ace1* gene shows improved disease conditions whereas, mice deficient in *Ace2* gene shows severe lung injury suggesting ACE2 as a key player in lung protection too.

The novel coronavirus 2019 (SARS-CoV2) has been shown to enter into human alveolar epithelial cells through mainly ACE2 (Zhou et al.

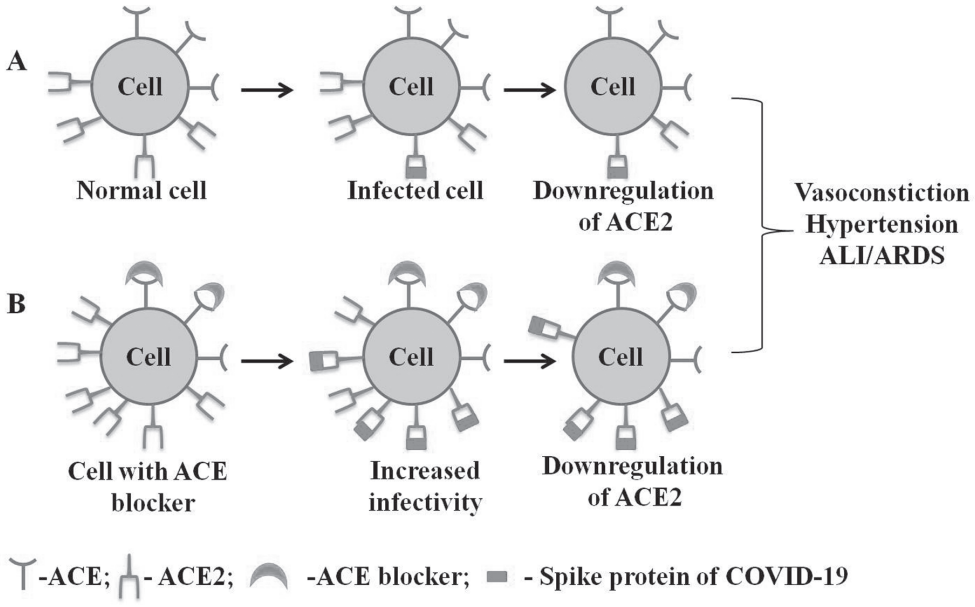


**Figure 1:** Schematic representation of the proposed mechanism of altered infectivity of COVID-19 with ACE and ACE2. A. Pathogenesis of COVID-19 in normal individuals leads to decreased ACE2 expression, B. Pathogenesis of COVID-19 in patients on ACE blockers leads to increased infectivity.

2020). As per the data released by the Chinese center for disease control and prevention, mortality of COVID-19 cases is directly related to being men, above 60 years of age, suffering from chronic diseases such as hypertension, diabetes, cardiovascular disease (Cheng et al. 2020; Wu et al. 2020).

It was found that SARS-CoV infection and SARS-CoV spike protein treatment to mice lead to downregulation of ACE2 expression in lung tissue (Kuba et al. 2005). As SARS-CoV2 spike protein had shown to have more similarity with SARS-CoV spike protein, we hypothesise that ACE2 could be down regulated in COVID-19 cases. Once the levels of ACE2 become low, increased ACE1 activity leads to vasoconstriction, hypertension and cardiac hypertrophy. More frequently hypertensive, diabetic, and/or having renal disease were observed in mortality cases of COVID-19 (Kuster et al. 2020).

Patients who are on ACE1 inhibitors and angiotensin II type-I receptor blockers had shown to upregulate ACE2 expression (Li et al. 2017). Thus, they are at higher risk for SARS-CoV2 infection as the expression of ACE2 is more, rate of infectivity of COVID-19 may be increased as ACE2 acts as a primary receptor (Figure 1).

Association studies of ACE polymorphism revealed that more DD genotype of ACE1 and G2350A may be associated with left ventricular hypertrophy (Fajar et al. 2019) and probably the severity of COVID-19. ACE2 polymorphism (rs4646188, rs2074192, rs4646155 rs4240157, rs4830542, rs879922 and rs2106809) is also shown to have association with hypertension (Patnaik et al. 2014; Pan et al. 2018).

Based on the available information, we propose to evaluate these polymorphisms which may have combined effect on infectivity, recovery and prognosis of COVID-19. Difference in the rate of infectivity and mortality of COVID-19 in various countries may be attributed to the ACE1 and ACE2 polymorphisms (Delanghe et al. 2020). To further evaluate this, a retrospective and prospective analysis of patients DNA can be analysed to find out the relationship so that the susceptible individuals may be treated accordingly.

**Conflict of interest**

No conflict of interest

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