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Editors

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An Observational Study to Assess the Prevalence and Awareness of Postpartum Depression among Females in Chandigarh and Surrounding Areas

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Abstract

Purpose: In females, postpartum depression may be due to abrupt hormonal changes post-pregnancy. We aimed to assess the prevalence of postpartum depression in females residing at Chandigarh and surrounding areas. Other objectives were to assess the awareness of postpartum depression in females and to determine the risk factors associated with postpartum depression.

Methods: A prospective observational study was carried out at selected hospitals and some part of data collection was done via online mode (survey). A total of 100 participants voluntarily took part in the investigation after signing the informed consent form. All of the data was statistically examined and presented.

Results: As per socio-demographic data, the majority of the participants were between the age of 20 and 30 years. Postpartum depression was observed to have a 70% overall prevalence, with participants reporting severe, moderate, and mild depression at rates of 18%, 35%, and 17%, respectively. Participants under the age of 40 were more knowledgeable about "postpartum depression" than those between 20 and 30. A significant correlation was found between postpartum depression and such factors as delivery type, unforeseen pregnancies, and despair during pregnancy ($p<0.222$).

Conclusion: We found a higher proportion of postpartum depression among moderately affected participants. Risk factors like delivery type, unwanted pregnancy, and depression during pregnancy should be addressed attentively in mothers with postpartum depression including follow-up at regular intervals.

Keywords: Postpartum depression, Risk factors, Age, Awareness.

Introduction

Postpartum depression is a kind of temper impairment that could have an effect on each parent following childbirth.[1] Its onset commonly takes place between 1 to 12 months after childbirth.[2] Some of the factors that contribute to the development of postpartum depression include hormone-related physiological changes social links to the records of depression, and new child-associated intimidating thoughts.[3] Hormones such as estrogen, progesterone, thyroid hormones, testosterone, corticotropin-releasing hormone, endorphins, and cortisol have all been linked to postpartum depression.[4] Estrogen and progesterone levels come back to what they were prior to pregnancy in about 24 hours upon giving birth, and this sudden shift might be the cause.[5] However, artificial oxytocin, a medication generally employed to help labor, has been connected to the progression of post-pregnancy despairing and uneasiness.[4] Several studies have shown that altered levels of gamma-aminobutyric acid (GABA) signaling, glutamate, serotonin, and dopamine play important roles in postpartum depression.[6]

In a study, it was found that globally, 20% of women suffer from postpartum depression after giving birth to a child.[7] In another similar kind of study, it was found that nearly 4,00,000 fathers are also dealing with this condition.[8] Postpartum psychosis, a more extreme type of post-pregnancy temperament illness, influences 1 to 2 out of each 1,000 females after labor.[9] In the United States, postpartum psychosis is one of the leading causes of child murder under the age of one year,[10] occurring in approximately 8 out of every 1, 00, 000 births. Although, it is a rare mental illness but should not be ignored.

The norms for diagnosing postpartum depression are like the ones for diagnosing essential despondency or minor melancholy that isn't connected all the time with labor. Within a two-week period, the criteria require at least five of the nine symptoms listed below:

1) Emptiness, sadness, or hopelessness almost every day for the majority of the day, or a depressed mood as observed by others
2) Anhedonia
3) Weight loss or a reduction in appetite
4) Change in sleep patterns
5) A sense of agitation
6) Energy depletion
7) Feelings of guilt or inadequacy
8) Concentration problems or increased hesitancy
9) Recurring thoughts of death, with or without suicidal intent[11]

Besides these, postpartum blues (a low temper that can loaf around for some days after giving birth) and postpartum psychosis are also considered for the diagnosis of postpartum depression.[2]

In order to treat mild to moderate postpartum depression, psychological therapies or antidepressants are employed. A combination of mental and medicinal healing procedures is much more likely to assist females with slight to excessive postpartum depression. For mild and moderate cases, light aerobic exercise has been found to be beneficial.[12] Exogenous oxytocin has only been examined in mice as a therapy for postpartum depression, but the findings seem encouraging for human use as well.[7] Exogenous oxytocin has only been studied in rodents as a postpartum depression treatment, but the results are promising for human application as well.[13] Some newer drugs like Brexanolone, a synthetic analog of the neurosteroid allopregnanolone, were approved by the Food and Drug Administration (FDA) for intravenous use in postpartum depression in 2019. Moreover, SAGE-217 and ganaxolone are two other new allopregnanolone analogs being studied for use in the treatment of postpartum depression.[7] We investigated this study to assess the prevalence and awareness of postpartum depression among females of different age groups along with the risk factors that may be responsible for the occurrence of post-pregnancy mental health illness.

Methodology

A prospective observational study was carried out at selected tertiary care hospitals located in Chandigarh and nearby areas. Some part of the study was conducted virtually with the help of an online survey. For doing so, an online questionnaire was generated and posted on various platforms in order to acquire the responses.

Before recruiting the subjects in the study, subjects were asked to satisfy the inclusion criteria. A total of 100 subjects voluntarily participated in the study after signing the informed consent form that included females with a newborn child/children and ones who were pregnant. This study was investigated for a period of 3 months after obtaining approval from the Institutional Ethics Committee (IEC). For data collection in person, a questionnaire was employed and responses were collected via one-to-one conversation.

An overall study design schema is illustrated in **Figure 1**.

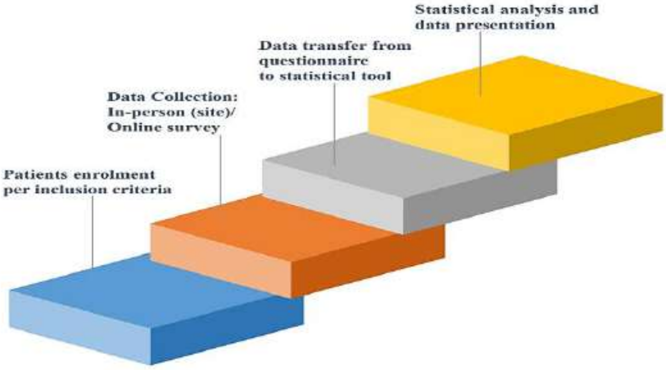

Figure 1 Study design procedures

Results

A total of 150 participants were screened. From these, 100 participants completed the questionnaire and overall study procedures. Based on the extensive information for the relevant questions, a significant volume of data was collected and analyzed.

Socio-demographic characteristics of the respondents

Socio-demographic characteristics of all the respondents are presented in **Table 1**. A majority of the participants were aged between 20-30 years (75%), had secondary and tertiary levels of education (50% each), and were unemployed (66%). Fifty-eight percent participants had unplanned pregnancies and 62% participants had premature deliveries.

Table 1 Demographic Characteristics of Participants

Characteristics Statistic	**Category**	**N=100 n (%)**
Age in years	20-30 years	75 (75%)
	30-40 years	23 (23%)
	40-50 years	1 (1%)
	50-60 years	1 (1%)
Education level	Primary	10 (10%)
	Secondary	50 (50%)
	Tertiary (College/University)	40 (50%)
Occupation	Unemployed	66 (66%)
	Employed	34 (34%)

Pregnancy planned	Planned	42 (42%)
	Unplanned	58 (58%)
Premature delivery	Yes	62 (62%)
	No	38 (38%)

N=Total number of subjects recruited.
n = No. of subjects with demographic characteristics

Prevalence and awareness about postpartum depression

The prevalence and severity of postpartum depression is presented in **Table 2**. The prevalence of postpartum depression was found to be 70% among females who participated in our study. Of the total participants, 18% were severely depressed, 35% were moderately depressed, and 17% had mild depression.

Table 2 Prevalence and Severity of Postpartum Depression

Severity of depression	**N = 100** **n (%)**
Participants with depression	70 (70%)
Mildly depressed	17 (17%)
Moderately depressed	35 (35%)
Severely depressed	18 (18%)
No depression	30 (30%)

N=Total number of subjects recruited.

n = No. of patients suffering from depression or no depression.

At post evaluation of study data on awareness about postpartum depression, study results revealed that a majority of the females (73%) were unaware of postpartum depression. A few females (27%) were familiar with the term "postpartum depression", while the remainder had never heard of it before being enrolled in the study (**Figure 2**).

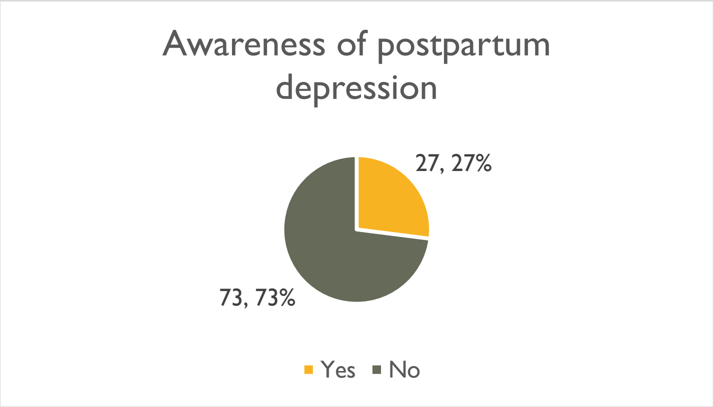

Figure 2 Awareness of Postpartum Depression

Association of age groups with awareness on postpartum depression

Participants between the ages of 30 and 40 were more aware of postpartum depression than those between the ages of 20 and 30 (87% vs 7%; **Table 3**).

Table 3 Association of Age Groups with Awareness on Postpartum Depression

Age-group	**Awareness on Postpartum depression**		**N=100**	**p-value**
	Yes	No		0.008

20 to 30 years	5 (7%)	70 (93%)	75	
30 to 40 years	20 (87%)	3 (13%)	23	
40 to 50 years	1 (100%)	0 (0)	1	
50 to 60 years	1 (100%)	0 (0)	1	
Total	27 (27%)	73 (73%)	100	

N=Total number of subjects recruited.

Risk Factors for Postpartum Depression

This study found that there were various risk factors associated with the occurrence of postpartum depression (**Table 4**). Of 62 (100%) participants with premature delivery, 51 (73%) participants were reported with postpartum depression. Of 51 (73%) participants with unintended pregnancies, 45 (64%) were reported with postpartum depression. Postpartum depression was found in 22 (31%) of the 31 (61%) participants with a history of depression. Additionally, 39 (56%) of the patients in the study exhibited postpartum depression and received no family support, while 57 (81%) reported depression during pregnancy.

Table 4 Risk Factors for Postpartum Depression

Associated risk factors	**Postpartum depression (N=100)**	
	Yes (n=70)	No (n=30)
Delivery Type		
Premature Delivery	51 (73%)	11 (37)
Mature Delivery	19 (27%)	19 (63%)
History of Depression		
Yes	22 (31%)	9 (30%)
No	48 (69%)	21 (70%)
Depression during pregnancy		
Yes	57 (81%)	10 (33%)
No	13 (19%)	20 (66%)
Unwanted pregnancy		
Yes	45 (64%)	6 (20%)
No	25 (36%)	24 (80%)
Family support		
Yes	31 (44%)	8 (27%)
No	39 (56%)	22 (73%)

N= Total number of subjects recruited.
n = No. of patients suffering from depression or no depression.

A significant correlation was found between postpartum depression and such factors as delivery type, unforeseen pregnancies, and despair during pregnancy ($p<0.222$).

Discussion

This observational study was carried out to assess the prevalence of postpartum depression and its awareness among females in Chandigarh and surrounding areas. In this study, we also investigated the risk factors associated with postpartum depression. The objectives of this study were hypothesized because of the support of the literature that implied females develop postpartum pregnancy after giving birth to their babies, although along-with assessment of the prevalence of postpartum awareness, it was also necessary to find the risk factors leading to it.[14, 15] Therefore, the questionnaire was tailored in order to assess the appropriateness of the study objectives. The subjects were selected after they fulfilled the consideration measures which were inclusive of enrolled females who had gone through pregnancy and may have encountered symptoms of postpartum depression.

A total of 42 subjects were engaged in a planned pregnancy while the remaining subjects had an unplanned pregnancy. The data of our study reported that the number of premature deliveries was greater than the normal deliveries. Females who had given birth prematurely were estimated at 66%. Premature deliveries might have a role in the development of subjects' postpartum depression.[16]

The outcomes of the study revealed that the prevalence among the severely depressed females was estimated at 28% compared to moderately depressed, mildly depressed, and no depression at 35%, 17%, and 20%, respectively.

With regard to the awareness of postpartum depression, it was found that only 27% of the total subjects enrolled in the study were aware of postpartum depression, and the rest of the subjects (73.7%) never heard of it. On the contrary, a similar study that was conducted recently reported over 50% of participants who had good knowledge of postpartum depression.[17] Furthermore, we discovered that women (20 participants) in the 30–40 year age range were more aware of postpartum depression than women (5 participants) in the 20–30 year age range. A scarcity of knowledge about how to cope with pregnancy and the appropriate steps to take in order to keep a pregnancy might be the reason.[18]

Data analysis also reported a significant relationship ($p<0.022$) between the age and the type of delivery experienced by the subject (premature or normal). Such a significant relationship was consistent with a recently conducted investigation.[19] The number of premature deliveries was found to be more in females who were in their 20s (63 cases) compared to those who were in their 30s (3 cases). Significant similarities were observed in the results of the study where females with young age ie, below their 20s, had more chances of preterm delivery than the females who were in their 30s. Moreover, the age group of 25 to 29 year was reported as the ideal range for avoiding preterm labor compared to females in their early 20s, and females with ≥35 years of age were associated with preterm delivery.[20] The results of our study suggest that the females in their 30s have more chances of normal deliveries than those in their 20s. Risk factors like delivery type, undesirable pregnancy, and depression during pregnancy were recognized as significantly connected with the postpartum depression status ($p<0.222$). These may have had a role in the event of their postpartum depression.[15, 21]

Conclusion

This study has found that postpartum depression is an important aspect associated with pregnancy. It was found to be more common in females under the age group of 20-30 years compared to 30-40 years. Our study reported an overall high prevalence of postpartum depression but higher proportion was noted among moderately affected participants. Certain risk factors like delivery type (premature), unwanted pregnancy, and depression during pregnancy may had played an important role in the development of postpartum depression. There should be a special need to identify and address all the associated risk factors in order to curb the progression of post-pregnancy mental health issues which may include both pharmacological (drug interventions) and non-pharmacological approaches (psychotherapeutic interventions).

Conflict of interest

The authors whose names are listed in the paper have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this paper.

Data availability statement

The data can be made available upon request from the author.

Ethics statement

This study was approved by the ethics committee. At the beginning of the study, participant was informed that the study was voluntary and they had the rights to quit at any time. The written informed consent was obtained from all individual participants included in this study.

Acknowledgement

Not applicable.

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Computational Identification of T-Cell Epitopes in Conserved Sequences Belonging to Genotype 6 of Hepatitis C Virus

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Abstract

Purpose: Hepatitis C Virus (HCV) is the leading cause of hepatocellular carcinoma and liver infections, which can be acute or chronic. For vaccine development, computational methods give us the best approaches for epitope-based vaccines against different types of viruses. In the early phase of acute hepatitis infection, only CD8+ and CD4+ T cells play a major role in fighting against HCV infection. In the current study, computational identification of multiple T-cell epitopes was done for the human genome with genotype 6.

Methods: Various immunoinformatics tools were used for the prediction of unique sequences of epitopes containing HCV glycoproteins, mainly E1 and E2 transmembrane proteins that show interaction with unique HLA allele sequences. The objective of the T-cell prediction is to find out the shortest sequence of antigen peptides that elicits the T cell immune response.

Results: Class I HLA alleles bound to the 8–11 amino acids, while Class II HLA bound to the 15–24 amino acids. The various tools used for epitope prediction of MHC class I CD8+ T cells were SYFPEITHI, NetCTL1.2, and IEDB. Similarly, the tools used for MHC class II CD4+ T cells were ProPred, MHC2 Pred, and IEDB for epitope prediction. Various unique HLA allele sequences were enlisted, for which various unique epitopes were predicted. A possible treatment for HCV is not known yet.

Conclusion: This study gives an approach to the future identification of vaccines in the case of HCV via in silico selection of epitopes, which can be further validated by in vivo and in vitro methods.

Keywords: Hepatitis C virus, Epitopes, HLA alleles, T-cells.

Introduction

The genetic structure and characteristics of HCV have been described well in the history.[1] The viral HCV could be a major blood-borne human infectious agent. There are currently 170 and 200 million cases of the virus worldwide, and 40,000 new cases are added each year. The HCV virus is an RNA-positive, positively polarized, 9500 nucleotide long virus.[2] It is considered as a significant public health issue since the virus is the etiological issue of chronic liver disease that often results in cirrhosis of the liver and malignant hepatoma (HCC). In developed countries, the foremost vital route of HCV transmission is blood vessel misuse, whereas in resource-poor countries, invasive procedures or injection-based therapies with contaminated instruments are the predominant sources of the latest infections.

Interferon-alpha and drugs like telaprevir and sofosbuvir can be used for the treatment of the HCV infection, but these therapies are costly.[3-4] Since people in developing countries can't afford such expensive treatments, there is a need to develop an affordable antiviral treatment. Thus, we need to gain knowledge about the epitopes of the virus for the advancement of the vaccine. At present, two vaccines are clinically used: one is developed by Chiron, which is recombinant protein E1 and E2, and the other, which makes CD8+ active, is made from the non-structural protein of the recombinant virus.[5]

Cytotoxic T cells (CTLs) are effective against the short sequences of peptide epitopes that are bonded with the human leucocyte antigen (HLA) and are expressed by the antigen-presenting cells. Through this, the evasion of HCV infection by immune responses could be avoided.[6]

Epitopes, also called antigen determinants, are the specific short peptide sequences in an antigen that stimulate the immune response when bound to the specific antibody. T-cell receptors (TCRs) are present on the surface of T cells and recognize the APC when bonded to the major histocompatibility complex (MHC). T-cell epitopes consist of two classes: MHC Class I and MHC Class II. CD4 cells bond to the MHC Class II through the receptors present on their surface, while CD8 cells bond with the MHC Class I. The MHC is present on chromosome 6, which codes for 21 genes and shows polymorphism. The three loci present on MHC Class I (A, B, and C) are only able to detect peptide sequences of eight to twelve amino acids in length. While Class II has HLA (DP, DQ, and DR) and detects amino acids of length eighteen to twenty-four, if we want to predict the T-cell epitopes, we must first know about the various HLA alleles.[7]

Various bioinformatics tools are available for the prediction of epitopes. Among this Class I bound with 8 to 11 AA, the C-terminal region and the 2nd position are known as anchor sites because these are important positions for the binding. A very limited number of proteins are acceptable for binding to the anchor site and binding to an HLA allele. Matrix-based methods are not able to correlate the relationship between the amino acids and how the binding affinity at one position of AA depends on the other amino acid position. In this study, we use three types of tools for class I prediction: SYFPEITHI, NetCTL1.2, and IEDB.

For Class II, prediction became difficult because both ends are open and allow more peptides to be bound, which shows higher polymorphism. Also, their binding motifs have weaker relativity. For their prediction, it was assumed that the 9 AA core motifs determined the affinity of the protein, MHC. This idea was used by TEPITOPE.[8]

In the early days, this was only for the use of PCs, but after a long time, this was provided as a part of the web server for the class II predictor, namely ProPred.[9] In this study, we have used IEDB, ProPred, and MHC2pred.

Epitope-based vaccines are becoming increasingly popular for the development of vaccines against viruses with conserved sequences or pathogens that have mutated rapidly. Using these various tools, appropriate in silico selection of epitopes could be possible.

Methods

Sequence retrieval

ViPR

ViPR stores information for virus families that are classified as either human priority pathogens or potential public health threats. ViPR works to combine data from three different sorts of sources: (i) data transmitted to ViPR from public archives; (ii) unique data created by ViPR utilizing a variety of computational algorithms and bioinformatics methodologies; and

(iii) data directly submitted to ViPR by independent researchers. In a relational database, these data are kept so that user-specified queries can quickly retrieve them.[10]

Whole genome sequences for E2 unique protein sequences were selected for downloading. Then, 121 protein sequences in FASTA format were retrieved for genotype VI.

Multiple sequence alignment

Multiple sequence alignment is a method for aligning more than two homologous nucleotide or amino acid sequences so that homologous residues from the respective sequences line up in columns as far as possible.

Clustal Omega

Clustal Omega is one of the bioinformatics tools used for aligning various multiple amino acid and nucleotide sequences precisely and rapidly. For decades, this has become one of the most used bioinformatics tools since it is a requirement for most phylogenetic or comparative studies of homologous genes or proteins.11 In this study, downloaded E2 protein sequences of Hepatitis C virus genotype 6 were used as input for Clustal Omega for multiple sequence alignment.

Conservancy analysis

AVANA

AVANA was further used to analyze the conserved region in the E2 protein after multiple sequence alignments through Clustal Omega. AVANA stands for Antigen Variability Analyzer Tool. This was used for the alignment of various subsets of the collected sequence that were entirely based on the viral subtype of the host. The tool provides useful information about the conserved region that is entirely based on information entropy analysis.[12] Information entropy is measured by the uncertainty of a random variable, or in the case of proteins, by protein mutation.[11] Highly conserved sites are shown due to lower entropy, which displays the degree of randomness.

To retrieve the conserved region of E2 protein, multiple sequence alignment of E2 protein in FASTA format was used by setting the parameter in AVANA as conservation region 70%, with a minimum length of 9 bp and a maximum length of 25 bp.

T-cell epitope prediction

Tools used for CLASS I MHC Epitope and HLA allele prediction

SYFPEITHI

The MHC databank is called SYFPEITHI. It regularly updates its database of MHC class I and class II ligands and peptide patterns from humans and other species, including apes, cattle, chicken, and mice, among others. There are distinct entries available for each motif that is currently available. It is possible to conduct searches for references, source proteins/organisms, T-cell epitopes, natural ligands, MHC alleles, and MHC motifs. Links are provided to the EMBL and PubMed databases.[13]

In this study, MHC-I prediction was performed by SYFPEITHI. To perform this task, all HLA were selected from HLA-A * 01 through HLA-DRB1 * 1501 (DR2b). Other parameters include the selection of nanomers, and 70% of the conserved sequence obtained from the above step was used as input. As a result, the HLA, epitope, and score appeared. The score must be less than or equal to 20.

NetCTL1.2

The NetCTL1.2 service is used to predict CTL epitopes in protein sequences. Version 1.2 of the MHC class I binding prediction now includes 12 MHC supertypes, including supertypes A26 and B39. In the technique, the efficiency of TAP transport, proteasomal C-terminal cleavage, and peptide MHC class I binding are all predicted. The service can be used to anticipate CTL epitopes that are restricted to 12 MHC class I supertypes. In order to achieve MHC class I binding and proteasomal cleavage, artificial neural networks are used. To forecast TAP transit effectiveness, use the weight matrix.[14] Subtype A1 was used to choose input from 70% of conserved sequences, starting with C1. The ordering by score was then altered to "Combination Score."

The remaining C1-C7 sequences were processed using the same procedures. The threshold for epitope recognition, the weight of C-terminal cleavage, and the weight of TAP transport effectiveness are all unchanged.

IEDB

Researchers from the La Jolla Institute for Allergy and Immunology (LIAI) have developed the Immune Epitope Database and Analysis Resource (IEDB) in collaboration with the Department of Health and Human Services (HHS) and the National Institute of Allergy and Infectious Diseases (NIAID). In order to generate new research tools, diagnostic techniques, vaccines, and therapies, immunological epitope data must be disseminated. Data curation for the ongoing NIAID Emerging and Re-emerging Infectious Diseases and Category A, B, and C priority pathogens (which includes influenza) is given top priority. Epitopes from diverse allergens, autoantigens, and infectious pathogens are also being catalogued. The database also contains information on immune epitopes from the TopBank, FIMM, HLA Ligand, and MHC binding databases, as well as information on MHC binding from a number of antigenic sources.[15]

C1-C7 conserved sequences were used as input, much as the prior technique. The ANN.4.0.0 prediction format was chosen. When you type HLA-A * 01:01, the outcome is shown. Afterward, choose HLA and epitope based on a score of 500 or fewer.

Tools used for CLASS II MHC epitope and HLA allele prediction

PROPRED

MHC class II binding sites in antigenic protein sequences were predicted using ProPred, a graphical online application. The server uses an amino-acid/position coefficient database derived from literature to create a matrix-based prediction method. In the graphical interface, the predicted binders may be seen as peaks, whereas in the HTML interface, they can be seen as colored residues. This website might help researchers find promiscuous binding areas that can connect to several HLA-DR alleles.[9]

Conserved sequences obtained from AVANA were used as input sequences. Select HLA alleles and epitopes on the basis of red and blue color. At a time, only nine sequences can be selected from ProPred.

MHC2 Pred

An SVM-based method is employed to predict the peptides that bind to MHC class II. SVM-based techniques are 80% accurate for 42 alleles.

Because the dataset was smaller, the method's performance was worse for a few alleles. The method's performance was evaluated using 5-fold cross-validation. MHC2Pred input sequences were 70% AVANA conserved sequences for the human allele HLA-DR1-HLA-

DRB5 * 0101, with a score of 0.5. The display mode would be tabular, and the tabular results would be 50. The results were then presented by HLA, epitope, and score. The score must be 0.5 or greater.

IEDB Analysis resource

MHC-2 prediction using IEDB was similar to one that we used for MHC-1 epitope prediction. By selecting human, HLA-DR, HLA-DP, and HLA-DQ species or loci one at a time. Then the results appear, with HLA, core sequence, peptide sequence, and score. Their selection was based on the score, which must be less than or equal to 500.

Results

Sequence retrieval

ViPR

In sequence retrieval, the unique sequence of 121 E2 proteins of the human genome had genotype 6, was retrieved in the FASTA format, and all had a unique sequence ID for each protein. The results for the sequence retrieval are shown in the **Table 5: A unique sequence of E2 protein in FASTA formatTable 5.**

Table 5: A unique sequence of E2 protein in FASTA format

S. No.	GenBank ID	Host	Country	Sequence ID
1	KC191671	Human	Malaysia	>gb:VIPR_ALG4_440234843_1468_2565
2	KJ567649	Human	Vietnam	>gb:VIPR_ALG4_685430775_1488_2594
3	KJ567648	Human	Vietnam	>gb:VIPR_ALG4_685430773_1489_2583
4	KJ567646	Human	Vietnam	>gb:VIPR_ALG4_685430769_1488_2588
5	KJ567651	Human	Vietnam	>gb:VIPR_ALG4_685430779_1488_2597
6	KJ567650	Human	Vietnam	>gb:VIPR_ALG4_685430777_1488_2594
7	KJ567652	Human	Vietnam	>gb:VIPR_ALG4_685430781_1488_2591
8	KJ567645	Human	Vietnam	>gb:VIPR_ALG4_685430767_1488_2582
9	KJ567644	Human	Vietnam	>gb:VIPR_ALG4_685430765_1473_2567
10	KJ567647	Human	Vietnam	>gb:VIPR_ALG4_685430771_1489_2580
11	KM504109	Human	-N/A	>gb:VIPR_ALG4_758739587_1489_2580
12	KJ678744	Human	China	>gb:VIPR_ALG4_758739124_1492_2595
13	KJ678745	Human	China	>gb:VIPR_ALG4_758739126_1492_2595
14	KM504110	Human	Thailand	>gb:VIPR_ALG4_758739589_1488_2576
15	KY014622	Human	China	>gb:VIPR_ALG4_AQT18930_1_1489_2583
16	JX183557	Human	China	>gb:VIPR_ALG4_407751085_1488_2585
17	KJ678746	Human	China	>gb:VIPR_ALG4_758739128_1449_2552
18	KJ678747	Human	China	>gb:VIPR_ALG4_758739130_1449_2552
19	KJ678748	Human	China	>gb:VIPR_ALG4_758739132_1429_2532
20	KJ678749	Human	China	>gb:VIPR_ALG4_758739134_1448_2551
21	KJ678750	Human	China	>gb:VIPR_ALG4_758739136_1449_2552
22	KJ678751	Human	China	>gb:VIPR_ALG4_758739138_1449_2552

S. No.	GenBank ID	Host	Country	Sequence ID
23	KJ678752	Human	China	>gb:VIPR_ALG4_758739140_1425_2528
24	KJ678753	Human	China	>gb:VIPR_ALG4_758739142_1449_2549
25	KJ678754	Human	China	>gb:VIPR_ALG4_758739144_1492_2595
26	KJ678755	Human	China	>gb:VIPR_ALG4_758739146_1449_2552
27	KJ678756	Human	China	>gb:VIPR_ALG4_758739148_1491_2594
28	KJ678757	Human	China	>gb:VIPR_ALG4_758739150_1491_2594
29	KJ678758	Human	China	>gb:VIPR_ALG4_758739152_1491_2594
30	KJ678759	Human	China	>gb:VIPR_ALG4_758739154_1491_2594
31	KJ678760	Human	China	>gb:VIPR_ALG4_758739156_1491_2594
32	KJ678761	Human	China	>gb:VIPR_ALG4_758739158_1491_2594
33	KJ678762	Human	China	>gb:VIPR_ALG4_758739160_1491_2594
35	KJ678764	Human	China	>gb:VIPR_ALG4_758739164_1491_2594
36	KJ678765	Human	China	>gb:VIPR_ALG4_758739166_1491_2594
37	KJ678766	Human	China	>gb:VIPR_ALG4_758739168_1449_2552
38	KJ678767	Human	China	>gb:VIPR_ALG4_758739170_1449_2552
39	KJ678768	Human	China	>gb:VIPR_ALG4_758739172_1446_2549
40	KJ678769	Human	China	>gb:VIPR_ALG4_758739174_1449_2552
41	KJ678770	Human	China	>gb:VIPR_ALG4_758739176_1449_2552
42	JX183549	Human	China	>gb:VIPR_ALG4_407751069_1488_2579
43	KM252792	Human	China	>gb:VIPR_ALG4_749396734_1488_2585
44	KM252793	Human	Laos	>gb:VIPR_ALG4_749396736_1488_2579
45	KM252794	Human	Laos	>gb:VIPR_ALG4_749396738_1488_2585
46	KM252789	Human	Laos	>gb:VIPR_ALG4_749396728_1491_2594
47	KM252795	Human	Laos	>gb:VIPR_ALG4_749396740_1488_2582
48	KM252796	Human	Laos	>gb:VIPR_ALG4_749396742_1488_2582
49	KM252797	Human	Laos	>gb:VIPR_ALG4_749396744_1474_2568
50	KM252790	Human	Laos	>gb:VIPR_ALG4_749396730_1491_2597
51	JX183554	Human	Laos	>gb:VIPR_ALG4_407751079_1488_2582
52	KM252798	Human	Laos	>gb:VIPR_ALG4_749396746_1491_2582
53	KM252799	Human	Laos	>gb:VIPR_ALG4_749396748_1491_2591
54	KM252800	Human	Laos	>gb:VIPR_ALG4_749396750_1488_2579
55	KM252791	Human	Laos	>gb:VIPR_ALG4_749396732_1491_2597
56	KM587629	Human	USA	>gb:VIPR_ALG4_751660982_1488_2585
57	KM587630	Human	USA	>gb:VIPR_ALG4_751660984_1488_2582
58	KM504112	Human	Canada	>gb:VIPR_ALG4_758739593_1488_2588
59	KM504113	Human	Canada	>gb:VIPR_ALG4_758739595_1489_2580
60	KM504114	Human	Canada	>gb:VIPR_ALG4_758739597_1488_2579

S. No.	GenBank ID	Host	Country	Sequence ID
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62	KJ678772	Human	Canada	>gb:VIPR_ALG4_758739180_1449_2552
63	KJ678773	Human	Canada	>gb:VIPR_ALG4_758739182_1449_2552
64	KM504115	Human	Canada	>gb:VIPR_ALG4_758739599_1489_2580
65	KM504116	Human	Canada	>gb:VIPR_ALG4_758739601_1489_2580
66	KM504118	Human	Canada	>gb:VIPR_ALG4_758739603_1488_2579
67	KJ678774	Human	Canada	>gb:VIPR_ALG4_758739184_1452_2555
68	KM504118	Human	Canada	>gb:VIPR_ALG4_758739605_1488_2579
69	KM504119	Human	Canada	>gb:VIPR_ALG4_758739607_1488_2570
70	KJ678775	Human	Canada	>gb:VIPR_ALG4_758739186_1449_2552
71	KM504120	Human	Canada	>gb:VIPR_ALG4_758739609_1488_2576
72	KM504121	Human	Canada	>gb:VIPR_ALG4_758739611_1489_2580
73	JX183558	Human	Canada	>gb:VIPR_ALG4_407751087_1488_2582
74	JX183550	Human	Canada	>gb:VIPR_ALG4_407751071_1488_2579
75	KM504122	Human	Canada	>gb:VIPR_ALG4_758739613_1488_2579
76	KJ678776	Human	Canada	>gb:VIPR_ALG4_758739188_1449_2552
77	KJ678777	Human	Canada	>gb:VIPR_ALG4_758739190_1449_2549
78	KM504124	Human	Canada	>gb:VIPR_ALG4_758739617_1489_2577
79	KJ678778	Human	Canada	>gb:VIPR_ALG4_758739192_1449_2552
80	KJ678779	Human	Canada	>gb:VIPR_ALG4_758739194_1449_2552
81	KJ678780	Human	Canada	>gb:VIPR_ALG4_758739196_1449_2549
82	KM504111	Human	Canada	>gb:VIPR_ALG4_758739591_1488_2579
83	KJ678781	Human	China	>gb:VIPR_ALG4_758739198_1492_2595
84	KJ678782	Human	China	>gb:VIPR_ALG4_758739200_1492_2595
85	KJ678784	Human	China	>gb:VIPR_ALG4_758739202_1492_2595
86	KJ678784	Human	China	>gb:VIPR_ALG4_758739204_1424_2527
87	KJ678785	Human	China	>gb:VIPR_ALG4_758739206_1449_2552
88	JX183551	Human	Vietnam	>gb:VIPR_ALG4_407751073_1488_2582
89	KM252779	Human	Vietnam	>gb:VIPR_ALG4_749396708_1488_2588
90	JX183555	Human	Vietnam	>gb:VIPR_ALG4_407751081_1488_2582
91	KM252780	Human	Vietnam	>gb:VIPR_ALG4_749396710_1488_2588
92	KM252787	Human	Vietnam	>gb:VIPR_ALG4_749396724_1488_2582
93	KM252786	Human	Vietnam	>gb:VIPR_ALG4_749396722_1488_2579
94	KM252782	Human	Vietnam	>gb:VIPR_ALG4_749396714_1489_2583
95	KM252783	Human	Vietnam	>gb:VIPR_ALG4_749396716_1474_2562
96	KM252785	Human	Vietnam	>gb:VIPR_ALG4_749396720_1489_2580
97	KJ678786	Human	Vietnam	>gb:VIPR_ALG4_758739208_1492_2595

S. No.	GenBank ID	Host	Country	Sequence ID
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99	KJ678788	Human	Vietnam	>gb:VIPR_ALG4_758739212_1491_2594
100	JX183552	Human	Vietnam	>gb:VIPR_ALG4_407751075_1488_2582
101	KJ678789	Human	Vietnam	>gb:VIPR_ALG4_758739214_1491_2594
102	KJ678790	Human	Vietnam	>gb:VIPR_ALG4_758739216_1449_2552
103	KJ678791	Human	-N/A-	>gb:VIPR_ALG4_758739218_1492_2595
104	KJ678792	Human	Vietnam	>gb:VIPR_ALG4_758739220_1492_2595
105	JX183556	Human	Vietnam	>gb:VIPR_ALG4_407751083_1488_2582
106	KM252782	Human	-N/A-	>gb:VIPR_ALG4_749396712_1488_2588
107	KJ678793	Human	Vietnam	>gb:VIPR_ALG4_758739222_1449_2552
108	KJ678794	Human	Vietnam	>gb:VIPR_ALG4_758739224_1492_2595
109	KJ678795	Human	Vietnam	>gb:VIPR_ALG4_758739226_1492_2595
110	KJ678796	Human	Vietnam	>gb:VIPR_ALG4_758739228_1449_2552
111	JX183553	Human	Vietnam	>gb:VIPR_ALG4_407751077_1488_2582
112	KM252788	Human	Vietnam	>gb:VIPR_ALG4_749396726_1489_2586
113	KJ678797	Human	Vietnam	>gb:VIPR_ALG4_758739230_1449_2552
114	KJ678798	Human	-N/A-	>gb:VIPR_ALG4_758739232_1449_2552
115	KM252784	Human	Vietnam	>gb:VIPR_ALG4_749396718_1487_2581
116	KC844040	Human	China	>gb:VIPR_ALG4_576294938_1488_2585
117	KC844039	Human	China	>gb:VIPR_ALG4_576294935_1488_2600
118	HQ912955	Human	China	>gb:VIPR_ALG4_324330491_1150_2253
119	HQ912954	Human	China	>gb:VIPR_ALG4_324330489_1150_2253
120	KC844037	Human	China	>gb:VIPR_ALG4_576294931_1448_2551
121	KC844038	Human	China	>gb:VIPR_ALG4_576294933_1448_2551

Multiple Sequence Alignment

Clustal Omega

The multiple sequence alignment shows the Consensus sequence of each unique 121 E2 protein sequence. The results for E2 protein sequences of HCV genotype 6 is shown below:

GENOTYPE VI

xxTTTVGxAVxRtTxGLxxLFSxGxKQNLQLINTNGSWHINRTALNCNDSLQTGFIASLF
YxHKFNSSGCPERMAACKPLxDFRQGWGxITYKxNISGPSDDRPYCWHYAPRPCxVV
PARTVCGPVYCFTPSPVVVGTTDRRGNPTYTWGENETDVFMLESLRPPxGGWFGCT
WMNSTGFTKTCGAPPCQIVPGDYNSxxxSxNELLCPTDCFRKHPEATYQRCGSGPWL
TPRCLVDYPYRLWHYPCTVNFTLHKVRMFVGGIEHRFDAACNWTRGERCELxDRDR
IEMSPLLFSTTQLAILPCSFTTMPALSTGLIHLHQNIVDVQYLYGVSSSVTSWVVKWE
YVVLMFLVLADARICTCLWLMLLIxxVEA

Conservancy analysis

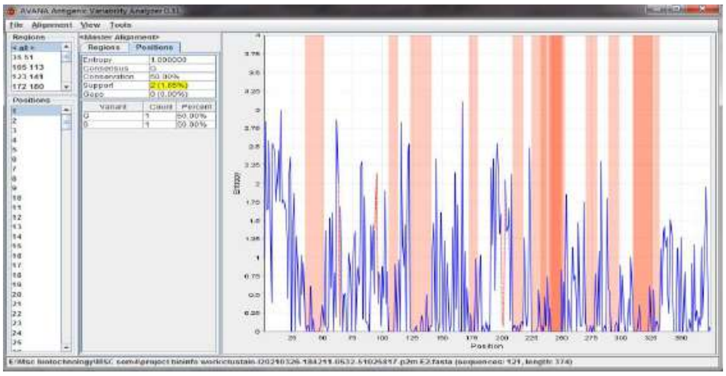
AVANA

The tool AVANA was used for the conservancy analysis. In this analysis, we took 70% of the conserved region as it shows the more unique sequence had a sequence length greater than 9. C1, C2, C3, C4, C5, C6, and C7 show the unique and non-repeated sequences with lengths greater than 9 (**Table 6** and **Figure 3**). This also represents the location of each sequence in the consensus sequence.

Table 6: 70% conserved region had unique sequence more than 9 length

Sr. No.	Sequence	Location
C1	NGSWHINRTALNCNDSL	35-51
C2	VCGPVYCFTPSPVVVGTTD	122-140
C3	CPTDCFRKHP	208-217
C4	CGSGPWLTPRCLVDYPYRLWHYPCTVNFT	224-252
C5	AACNWTRGERCE	269-280
C6	EMSPLLFSTTQ	288-298
C7	TMPALSTGLIHLHQNIVDVQYLYG	308-331

Figure 3: Orange color shows the 70% conserved region by using AVANA



Entropy 1.000000
Consensus G
Conservation 50.00%
Support 2 (1.65%)
Gaps 0 (0.00%)

Epitope prediction

It was done for both MHC class I and II with different tools. The results of these tools are mentioned below based on their category and tools.

MHC Class I

In class I epitope prediction, epitopes were predicted for conserved sequences C1, C2, C4, and C7 by using the tools SYFPEITHI and NetCTL1.2 (**Table 3**). In the case of the IEDB epitope, it was predicted for conserved sequences C2, C4, C6, and C7. The SYPEITHI tool had predicted 1 epitope for C1, 2 epitopes for C2, 10 for C4, and 7 for C7. Similarly, NetCTL1.2 predicted 1 epitope for C1, 2 epitopes for C2, 10 for C4, and 6 for C7, while 1 epitope for C2, 7 epitopes for C4, 1 epitope for C5, 1 epitope for C6, and 6 epitopes for C7 were predicted by IEDB (**Table 7**).

Table 7: Epitopes predicted by SYEPEITHI, NetCTL1.2, and IEDB tools for MHC I

Conserved Sequence	SYEPEITHI	NetCTL1.2	IEDB
C1	TALNCNDSL	SWHINRTAL	Not applicable
C2	TPSPVVVGT, CFTPSPVVV	VYCFTPSPV,GPVYCFTPS	VYCFTPSPV

Conserved Sequence	SYEPEITHI	NetCTL1.2	IEDB
C4	LTPRCLVDY,RLWHYPCTV,CLVDYPYRL,WLTPRCLVD,GSGPWLTPR, DYPYRLWHY,GPWLTPRCL,WHYPCTVNF,PRCLVDYPY LVDYPYRLW	LTPRCLVDY,RLWHYPCTV,CLVDYPYRL,DYPYRLWHY,HYPCTVNFT, DYPYURLWHY,GPWLTPRCL,YRLWHYPCT,PRCLVDYPY WHYPCTVNF	RLWHYPCTV,GSGPWLTPR,CLVDYPYRL,WHYPCTVNF, RCLVDYPYR,GPWLTPRCL,LVDYPYRLW
C5	Not applicable	Not applicable	ACNWTRGER
C6	Not applicable	Not applicable	EMSPLLFST
C7	ALSTGLIHL,HLHQNIVDV,GLIHLHQNI LIHLHQNIV,TMPALSTGL,HQNIVDVQY MPALSTGLI	NIVDVQYLY,HQNIVDVQY,ALSTGLTHL,TMPALSTGL ,HLHQNIVDV,MPALSTGLI	NIVDVQYLY,ALSTGLIHL,MPALSTGLI,TMPALSTGL, HQNIVDVQY,GLIHLHQNI

MHC Class II

In class II epitope prediction, epitopes were predicted for conserved sequences C1, C2, C4, and C7 by using ProPred, while MHC2Pred predicted epitopes for all conserved sequences C1, C2, C3, C4, C5, C6, and C7. Whereas in the case of IEDB, it was predicted for conserved sequences C1, C2, C4, C5, C6, and C7. ProPred predicted 1 epitope for C1, 3 for C2, 3 for C4, and 3 for C7 as well. MHC2Pred predicted 9 epitopes for C1, 11 for C2, 2 for C3, 21 for C4, 5 for C5, 3 for C6, and 14 for C7. IEDB predicted 4 epitopes for C1, 12 for C2, 16 for C4, 2 each for C5, and C6, and 16 for C7 **(Table 8**).

As compared to MHC class I tools, MHC class II tools have predicted epitopes for more conserved sequences.

Table 8: Epitopes predicted by ProPred, MHC2Pred, and IEDB tools for MHCII

Conserved Sequence	ProPred	MHC2Pred	IEDB
C1	WHINRTALN	NGSWHINRT,INRTALNCN,NRTALNCND,WHINRTALN, GSWHINRTA,TALNCNDSL,HINRTALNC , SWHINRTAL, RTALNCNDS	WHINRTALN,GSWHINRTA,INRTALNCN,SWHINRTAL
C2	VYCFTPSPV ,FTPSPVVVG, YCFTPSPVV	PSPVVVGTT,CGPVYCFTP,SPVVVGTTD,FTPSPVVVG, CFTPSPVVV,YCFTPSPVV,TPSPVVVGT,VCGPVYCFT,GPVYCFTPS PVYCFTPSP,VYCFTPSPV	YCFTPSPVV,VYCFTPSPV,CFTPSPVVV,FTPSPVVVG,VCGPVYCFT,PVYCFTPSP TPSPVVVGT,YCFTPSPVX,GPVYCFTPS,SPVVVGTTD,CGPVYCFTP,PSPVVVGTT
C3	Not applicable	CPTDCFRKH,PTDCFRKHP	Not applicable
C4	WHYPCTVNF ,YRLWHYPCT, YRLWHYPC	PWLTPRCLV,CGSGPWLTP,HYPCTVNFT,LWHYPCTVN,WLTPRCLVD, LVDYPYRLW,TPRCLVDYP,RLWHYPCTV,LTPRCLVDY,GP	YPYRLWHYP,CLVDYPYRL,PYRLWHYPC,LVDYPYRLW,VDYPYRLWH, LWHYPCTVN,RLWHYPCTV, YRLWHYPCT,WHYPCTVNF,

Conserved Sequence	ProPred	MHC2Pred	IEDB
		WLTPRCL,	PWLTPRCLV,
		SGPWLTPRC,CLVDYPYRL,DYPYRLWHY,RCLVDYPYR,WHYPCTVNF	RCLVDYPYR,CGSGPWLTP,LTPRCLVDY,WLTPRCLVD,PRCLVDYPY
		GSGPWLTPR,PRCLVDYPY,YPYRLWHYP,PYRLWHYPC,VDYPYRLWH,	HYPCTVNFT
		YRLWHYPCT	
C5	Not applicable	NWTRGERCE,CNWTRGERC,NWTRGERC,EACNWTRGER,AACNWTRGE	ACNWTRGER,CNWTRGERC
C6	Not applicable	MSPLLFSTT,EMSPLLFST,SPLLFSTTQ	EMSPLLFST,MSPLLFSTT
C7	LIHLHQNIV, LHQNIVDVQ, IHLHQNIVD	STGLIHLHQ,LSTGLIHLH,HQNIVDVQY,QNIVDVQYL,LHQNIVDVQ,MPALSTGLI	LIHLHQNIV,LSTGLIHLH,IHLHQNIVD,ALSTGLIHL,HLHQNIVDV,GLIHLHQNI
		HLHQNIVDV,IVDVQYLYG,TMPALSTGL,NIVDVQYLY,TGLIHLHQN,PALSTGLIH	MPALSTGLI,LHQNIVDVQ,PALSTGLIH,NIVDVQYLY,TGLIHLHQN,TMPALSTGL
		LIHLHQNIV,IHLHQNIVD,ALSTGLIHL,GLIHLHQNI	IVDVQYLYG,HQNIVDVQY,QNIVDVQYL,STGLIHLHQ

HLA analysis

MHC Class I

In class I HLA allele prediction, HLA alleles were predicted for conserved sequences C1, C2, C4, and C7 by tools SYFPEITHI and NetCTL 1.2. But in the case of IEDB, HLA alleles are predicted for C2, C4, C5, C6, and C7. SYFPEITHI predicted 1 HLA allele for C1, 2 for C2, 15 for C4, 4 for C7, whereas NetCTL1.2 predicted 3 HLA alleles for C1, 2 for C2, 6 for C4, 5 alleles for C7. IEDB predicted 1 HLA allele for C2, 12 for C4, 1 for C5, 2 for C6, and 12 for C7 **(Table 9)**.

Table 9: HLA alleles predicted by SYFPEITHI, NetCTL1.2, and IEDB tools for MHC class I

Conserved Sequence	SYFPEITHI	NetCTL1.2	IEDB
C1	HLA-B*51:01	A24,B8,B39	Not applicable
C2	HLA-B*07:02 ,HLA-B*15:16	A24,B7	HLA-A*23:01
C4	HLA-A*01,HLA-A*02:01,HLA-A*03 ,HLA-A*11:01,HLA-A*26 , HLA-A*68:01 ,HLA-B*07:02,HLA-B*13 ,HLA-B*15:10 HLA-B*15:16 ,HLA-B*27:05 ,HLA-B*35:01	A1,A2,A24,A26,B27,B39	HLA-A*02:01,HLA-A*02:02,HLA-A*02:03 HLA-A*02:04,HLA-A*02:05,HLA-A*02:06 HLA-A*02:07,HLA-A*02:08,HLA-A*02:09 HLA-A*02:10,HLA-A*02:11,HLA-A*02:12

Conserved Sequence	SYFPEITHI	NetCTL1.2	IEDB
	HLA-B*38:01 ,HLA-B*51:01 ,HLA-B*58:02		
C5	Not applicable	Not applicable	HLA-A*31:01
C6	Not applicable	Not applicable	HLA-A*02:06,HLA-A*68:02
C7	HLA-A*02:01 ,HLA-B*13 ,HLA-B*15:01 (B62) ,HLA-B*51:01	A1,A2,A26,B7,B62	HLA-A*29:02,HLA-A*02:01,HLA-A*02:06 HLA-A*68:01,HLA-B*07:02,HLA-B*53:01 HLA-A*26:01,HLA-A*30:02,HLA-B*51:01 HLA-B*15:01,HLA-B*35:01,HLA-A*68:02

MHC Class II

In the case of Class II HLA prediction, HLA alleles were predicted for conserved sequences C1, C2, C4, and C7 by using the tool ProPred. In MHC2Pred, it was predicted for conserved sequences C1, C2, C3, C4, C5, C6, and C7 and in the case of IEDB, the HLA allele was predicted for conserved sequences C1, C2, C4, C5, C6, and C7. ProPred predicted 2 HLA alleles for C1, 8 for C2, 6 for C4, and 14 for C7. MHC2Pred predicted HLA allele 17 for C1, 16 for C2, 5 for C3, 22 for C4, 5 for C5, 9 for C6, and 23 for C7. The IEDB tool predicted 17 HLA alleles for C1, 32 for C2, 38 for C4, 3 for C5, 4 for C6, and 35 for C7. The maximum HLA alleles were predicted by the IEDB tool for MHCII as compared to MHCI **(Table 10).**

Table 10: HLA alleles predicted by ProPred, MHC2Pred, and IEDB tools for MHC II

Conserved Sequence	ProPred	MHC2Pred	IEDB
C1	DRB1_0305,DRB1_0309	HLA-DR9,HLA-DR2,HLA-DR3,HLA-DR51 HLA-DQ4,HLA-DQ6,HLA-DQ7,HLA-DQ8 HLA-DQB1*03,HLA-DQB1*0301,HLA-DRB1*0101 HLA-DRB4*0101,HLA-DRB1*0301,HLA-DRB1*0405 HLA-DRB1*0901,HLA-DRB1*1101,HLA-DRB1*1501	HLA-DRB3*02:02,HLA-DQA1*02:01/DQB1*04:02 HLA-DQA1*03:03/DQB1*04:02 HLA-DQA1*06:01/DQB1*04:02 HLA-DRB1*04:05,HLA-DRB1*11:01 HLA-DRB1*08:01,HLA-DQA1*05:01/DQB1*04:02 HLA-DRB3*03:01,HLA-DQA1*01:02/DQB1*06:02 HLA-DQA1*01:02/DQB1*05:01 HLA-DRB1*13:02,HLA-DRB1*07:01 HLA-DRB1*01:01,HLA-DRB1*04:04 HLA-DRB1*13:01,HLA-DRB4*01:03
C2	DRB1_0309,DRB1_030	HLA-DR9,HLA-DR2,HLA-	HLA-DRB1*07:01,HLA-

Conserved Sequence	ProPred	MHC2Pred	IEDB
	9,DRB1_0421 DRB1_0423,DRB1_0701,DRB1_0703 DRB1_1501,DRB1_1506	DR3,HLA-DR13 HLA-DR15,HLA-DR52,HLA-DQ4,HLA-DQ6 HLA-DQ7,HLA-DQ8,HLA-DQB1*0301 HLA-DRB1*0101,HLA-DRB5*0101,HLA-DRB1*0401 HLA-DRB1*0405,HLA-DRB1*0901	DRB1*09:01 HLA-DQA1*05:01/DQB1*03:03 HLA-DQA1*06:01/DQB1*04:02 HLA-DQA1*02:01/DQB1*04:02 HLA-DQA1*05:01/DQB1*03:02,HLA-DRB3*02:02 HLA-DQA1*05:01/DQB1*04:02 HLA-DQA1*02:01/DQB1*03:03 HLA-DQA1*02:01/DQB1*03:01,HLA-DRB3*03:01 HLA-DQA1*01:03/DQB1*06:03,HLA-DRB1*04:01 HLA-DRB1*04:04,HLA-DPA1*01:03/DPB1*02:01 HLA-DRB1*13:02,HLA-DRB1*04:05 HLA-DQA1*05:01/DQB1*03:01 HLA-DRB1*16:02,HLA-DRB1*01:01 HLA-DRB1*08:02,HLA-DPA1*01:03/DPB1*06:01 HLA-DRB1*10:01,HLA-DQA1*03:01/DQB1*03:01 HLA-DRB1*13:01,HLA-DQA1*01:02/DQB1*05:01 HLA-DRB4*01:03,HLA-DRB1*08:01 HLA-DRB1*15:01,HLA-DRB1*11:01 HLA-DRB3*01:01,HLA-DPA1*02:01/DPB1*14:01
C3	Not applicable	HLA-DR9,HLA-DQ4,HLA-DQ7,HLA-DRB1*0405 HLA-DRB1*0901	Not applicable
C4	DRB1_0309,DRB1_0405,DRB1_0421 DRB1_0703,DRB1_1502,DRB1_1506	HLA-DR4,HLA-DR9,HLA-DR2,HLA-DR3 HLA-DR8,HLA-DR13,HLA-DR15 HLA-DR52,HLA-DQ4,HLA-DQ6 HLA-DQ7,HLA-DQ8,HLA-	HLA-DQA1*05:01/DQB1*04:02,HLA-DRB3*01:01 HLA-DPA1*01:03/DPB1*06:01 HLA-DPA1*01:03/DPB1*02:01,HLA-DRB3*03:01 HLA-DRB1*15:01,HLA-

Conserved Sequence	**ProPred**	**MHC2Pred**	**IEDB**
		DQB1*0301 HLA-DRB4*0101,HLA-DRB5*0101 HLA-DRB1*0301,HLA-DRB1*0401 HLA-DRB1*0405,HLA-DRB1*0802 HLA-DRB1*0901,HLA-DRB1*1501, HLA-DRB5*0101	DPA1*03:01/DPB1*04:02 HLA-DQA1*01:01/DQB1*05:01,HLA-DRB1*03:01 HLA-DRB4*01:03,HLA-DRB1*09:01 HLA-DRB1*04:04,HLA-DRB1*16:02 HLA-DPA1*01:03/DPB1*03:01,HLA-DRB1*13:01 HLA-DRB1*04:05,HLA-DQA1*02:01/DQB1*04:02 HLA-DRB1*10:01,HLA-DPA1*02:01/DPB1*01:01 HLA-DQA1*01:02/DQB1*05:01,HLA-DRB1*01:01 HLA-DPA1*01:03/DPB1*04:01 HLA-DQA1*02:01/DQB1*03:01,HLA-DRB1*04:02 HLA-DRB1*07:01,HLA-DRB1*13:02,HLA-DRB1*12:01,HLA-DPA1*02:01/DPB1*05:01 HLA-DQA1*03:01/DQB1*03:01 HLA-DQA1*03:03/DQB1*04:02,HLA-DRB5*01:01 HLA-DRB1*08:02,HLA-DQA1*01:02/DQB1*05:02 HLA-DQA1*06:01/DQB1*04:02,HLA-DRB1*08:01 HLA-DRB3*02:02,HLA-DQA1*05:01/DQB1*03:01 HLA-DQA1*05:01/DQB1*03:03
C5	Not applicable	HLA-DR4,HLA-DR9,HLA-DRB1*0301 HLA-DRB1*0405,HLA-DRB1*0901	HLA-DRB1*13:01,HLA-DRB1*07:01 HLA-DRB4*01:03
C6	Not applicable	HLA-DR9,HLA-DQ4,HLA-DQ7,HLA-DQ8, HLA-DQB1*0301,HLA-DRB1*0401 HLA-DRB1*0405,HLA-	HLA-DRB4*01:03,HLA-DQA1*01:02/DQB1*05:01 HLA-DQA1*05:01/DQB1*03:02 HLA-DPA1*01:03/DPB1*06:01

Conserved Sequence	**ProPred**	**MHC2Pred**	**IEDB**
		DRB1*0901 HLA-DRB1*1501	
C7	DRB1_0102,DRB1_0306,DRB1_0307 DRB1_0308,DRB1_0311,DRB1_0401 DRB1_0402,DRB1_0405,DRB1_0410 DRB1_0421,DRB1_0426,DRB1_1107 DRB1_1304,DRB1_1506	HLA-DR4,HLA-DR9,HLA-DR2 HLA-DR7,HLA-DR13,HLA-DR15 HLA-DR52,HLA-DR53,HLA-DQ4 HLA-DQ6,HLA-DQ7,HLA-DQ8 HLA-DQB1*03,HLA-DQB1*0301 HLA-DRB1*0101,HLA-DRB4*0101 HLA-DRB1*0301,HLA-DRB1*0401 HLA-DRB1*0405,HLA-DRB1*0802 HLA-DRB1*0901,HLA-DRB1*1101 HLA-DRB1*1501	HLA-DRB3*03:01,HLA-DRB1*13:01 HLA-DRB1*01:01,HLA-DPA1*03:01/DPB1*04:02 HLA-DQA1*01:02/DQB1*05:01,HLA-DRB1*04:04 HLA-DRB4*01:01,HLA-DRB1*13:02 HLA-DQA1*05:01/DQB1*03:03 HLA-DQA1*02:01/DQB1*03:01 HLA-DRB4*01:03,HLA-DQA1*01:02/DQB1*05:02 HLA-DPA1*01:03/DPB1*06:01,HLA-DRB1*07:01 HLA-DQA1*03:01/DQB1*03:01 HLA-DRB1*10:01,HLA-DRB3*02:02 HLA-DQA1*01:02/DQB1*06:02 HLA-DPA1*02:01/DPB1*01:01,HLA-DRB1*04:05 HLA-DQA1*02:01/DQB1*03:03 HLA-DQA1*01:01/DQB1*05:01,HLA-DRB1*16:02 HLA-DRB1*12:01,HLA-DQA1*05:01/DQB1*03:01 HLA-DRB1*15:01,HLA-DQA1*05:01/DQB1*03:02 HLA-DRB1*09:01,HLA-DQA1*02:01/DQB1*04:02 HLA-DPA1*01:03/DPB1*02:01,HLA-DRB1*04:02 HLA-DRB1*04:01,HLA-DQA1*05:01/DQB1*04:02 HLA-DRB1*03:01,HLA-DQA1*05:01/DQB1*02:01

Discussion

There is identification of the requirement to stimulate each arm of the adaptive reaction for an efficient preventative HCV immunogen and proof to support the inclusion of each structural and non-structural protein.[16] Previous studies have shown proof of cross-reactive neutralizing antibodies (NAbs), significantly within the great ape model.[17-19] However, they have restricted knowledge on immunogen candidates that elicit neutralizing and HCV-specific T-lymphocyte responses.

Some positive results have been shown in a research on African monkeys and immunized mice by mixing the glycoproteins E1, E2, non-structural protein 3 (NS3), and the core protein in the alum (consisting of conserved T-cell epitopes) for the induction of sensitive neutralizing antibodies and wide cellular responses (CD4+ and CD8+).[20] Few vectors and DNA-based vaccines were also present, but results from the phase 2 trial showed that they increased productivity because they were likely to be in an "at-risk' population.[14]

In the relatively large and currently thirty-year field of HIV immunizing agent development, the dearth of an efficient immunizing agent points to the various remaining barriers to inducement, generally neutralizing antibodies or effective CD8+ T cells capable of acting and continuous at the positioning of tissue layer HIV entry. Several current immunizing agent methods going into clinical studies look to handle a number of the evasion mechanisms mentioned here. For instance, there has been testing of assorted diversity-combating antigen-style approaches as well as mosaic vaccines, during which the inclusion of variant epitopes is optimized, as well as ways to preserve immunogen sequences. There are varied adjuvants, vectors, and delivery vehicles designed to boost the potency of the presentation of immunizing agent antigens so as to stimulate effective antiviral responses. A unique "tolerogenic" immunizing agent consisting of inactivated simian immunological disorder virus (SIV) mac239 particles with explicit microorganism adjuvants has been shown to elicit CD8+ T-regulatory cells in immunized macaques.

T lymphocytes were not lysed but were ready to suppress the activation of SIV-positive CD4+ T cells, making them less prone to SIV infection once challenged. Additionally, these CD8+ T cells were found to have unusual resistance provided by non-classical MHCIb/E molecules[20], such as HLA-E in humans. Curiously, recent knowledge shows HLA-E expression in liver biopsies correlates with HCV microorganism load in chronic HCV-infected subjects, and NK cells lacking the repressive receptor for HLA-E (NKG2A) are related to protection from HCV infection in bad-exposure subjects.[21] Thus far, there has been no examination of non-classical HLA-restricted CD8+ T cells in HCV infection. In distinction to the CD8+ "T-regulatory" sort cells represented above, an immunizing agent supported by a rhesus herpes virus vector has made sturdy protection or clearance of SIV challenge infections in unsusceptible macaques related to the induction of effector memory CD8+ T lymphocyte responses. However, these CD8+ T cells are found to focus on a variety of promiscuous or dominant epitopes restricted by HLA category II alleles instead of HLA category I.[22-23]

Conclusion

In this study, computational identification of multiple T cell epitopes was done for genotype 6 of the human genome with the help of various epitope prediction tools. The tools used for MHC class I epitope prediction were SYFPEITHI, NetCTL1.2, and IEDB for CD8+ T cells. Similarly, for MHC class II epitope prediction, the tools were ProPred, MHC2Pred, and IEDB for CD4+ T cells. These tools give unique epitope sequences. However, various unique HLA allele sequences were enlisted, for which the epitopes were predicted. This study gives the platform to identify future Hepatitis C vaccine candidates for genotype 6 for further in silico and experimental validation in vivo and in vitro cultures.

Conflict of interest

The authors whose names are listed in the paper have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the paper's subject matter or accoutrements.

Data availability statement

The data can be made available upon request from the author.

Ethics statement

Not applicable.

Acknowledgement

Not applicable.

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Screening of Protease-producing Fungi from Soil Samples by Conventional and Molecular Methods

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Abstract

Purpose: To isolate protease-producing fungi from soil samples by conventional and molecular methods. Other objectives included comparing isolated fungi for protease production and identifying them molecularly using 18s RNA sequencing.

Methods: The soil samples were rich in organic matter and were used for the isolation of proteolytic fungi. The soil samples were collected from three sites, viz., gardens, crop fields, and lakes, in different localities of Punjab and Chandigarh. The molecular identification of the fungus was carried out using 18s RNA sequencing after the purification and identification of isolated fungi.

Results: During the study, it was found that a total of 9 samples were found positive for the occurrence of fungi. The percentage frequency of positive samples in different areas was found to be 50%, 33.3%, and 50% in samples collected from crop fields, garden soil, and lake soil. In the present study, from the 9 positive fungal samples, a total of 3 fungal forms were obtained that produce the protease enzyme. These were identified and found to belong to *Alternaria* and *Penicillium sp.* The samples belonging to garden soil yielded only one fungal species, *Alternaria solani*, and the samples of lake soil yielded *Cladosporium sp.* A total of two *Penicillium sp.* have been isolated from Mohali samples.

Conclusion: The isolated strain of *Penicillium sp.* was found to be a potential producer of extracellular protease rather than *Alternaria*. The enzyme yield was greater in *Penicillium sp.* than in *Alternaria sp.* The findings highlight the importance of a local isolate of *Penicillium sp.* because it is particularly good at producing extracellular protease during fermentation.

Keywords: Fungi, Protease, Soil, *Penicillium*, *Alternaria.*

Introduction

The soil is a dynamic medium for microbial and biological activities, and the number and kind of microorganisms present in a particular soil depend on many environmental factors, such as the amount and type of available nutrients, moisture, the degree of aeration, pH, temperature, etc.[1] They play a pivotal role in various biochemical processes and, thus, are responsible for recycling organic compounds in nature.[2]

Fungi are an important part of the microbiota, and they have a tendency to decompose various types of organic materials, consequently regulating the ratio of nutrients and carbon in the soil.[3] It is estimated that there are 1.5 million fungal species on earth, of which only 74,000 have been described up to now, which presents a potential source of novel organisms.[4]

Enzymes are among the most significant products from microbial sources for human requirements. Recent advances in biotechnology are enabling enzymes to find new uses. A protease is an enzyme that initiates protein degradation by hydrolyzing the peptide bonds that connect amino acids in a polypeptide chain to produce the protein. Proteases work best in acidic conditions.[5]

As obligatory heterotrophs, fungi naturally break down organic matter and produce a variety of enzymes. Fungal enzymes comprise more than half of industrial enzymes and are effectively utilized in various industrial processes and products. Pulp and paper, clothing, detergents, food, feeds, nutraceuticals, and pharmaceuticals are well-known industries. *Aspergillus* is the most widely used fungus genus in manufacturing industrial enzymes. Other than protease, phytase, L-asparaginase, and a few others, glycosyl hydrolases (such as cellulases, xylanases, mannanases, amylase, pectinases, and others) make up the majority of commercial fungal enzymes.[6]

A study demonstrated that the proteolytic fungus could be isolated from soil samples and grown on Reese agar plates with casein as the protein substrate. An investigation was carried out in the various regions of the Madhya Pradesh districts of Sagar and Jabalpur. There were 50 soil samples obtained, and a total of 38 of those samples tested positive for the presence of fungi. The positive soil samples yielded 141 different fungus types. These comprised 38 from crop field soils, 47 from garden soil, and 56 from poultry farms' fungi. *Fusarium* and *Aspergillus* were the two most common genera.[7] In a different study, microorganisms that produce proteases were identified in soil samples. *Aspergillus*, *Rhizopus*, *Fusarium*, and *Mucor sp.* were the isolates that produced a high percentage of positive screening results. *Aspergillus sp.* 14L3S, an isolate from the soil surface, has the highest protease activity of these isolates, as shown by the clear zone surrounding the colony. According to the findings of these investigations, fungi have immense potential to be used in commercial manufacturing and for the production of protease enzymes.[8]

Another study showed that the amount of fungi in the soil was isolated using the soil-plate method. Researchers used this method to show that fungi can be found in various environments, including rhizosphere soils.[9] Proteolytic fungi were recovered from a variety of sources, including garden soil, alkaline soil, and soil samples.[10] In Coimbatore, Tamil Nadu, Kalpana Devi isolated a proteolytic *Aspergillus niger*.[11] Charles et al. isolated *Aspergillus nidulans* HA-10, a proteolytic fungus, from poultry farm soil.[12]

The main focus of the present study was to isolate the protease-producing fungi from soil samples by conventional and molecular methods. The other objectives included comparing isolated fungi for protease production and identifying them molecularly using 18s RNA sequencing.

Methodology

Sample collection

The soil samples were rich in organic matter and were used to isolate proteolytic fungi. The soil samples were collected from three sites, viz., gardens, crop fields, and lakes, in different localities of Punjab and Chandigarh. The samples were randomly collected from the above habitats from the superficial layer of soil not exceeding 5-6 cm in depth using a pre-sterilized spatula. Then, the soil was transferred into sterilized polythene bags. The samples were then brought to the laboratory and kept at 4˚C until processed. The general study design and sample collection are detailed in Error! Reference source not found..

Determination of pH of the soil samples

To check the pH of the soil samples, 10 g of each soil sample was suspended in 100 mL of double-distilled water and shaken vigorously for 30 minutes. The pH was then checked using a pH meter.

Screening of fungi from the soil sample

Sabouraud dextrose agar (SDA) medium and potato dextrose agar (PDA) medium were used as growth media. Initially, the pH of the medium was adjusted to 9.0 with a solution of 1 N NaOH. The serial dilution method isolated the fungal strain from the soil[13] on SDA and PDA media. Then, 1 g (dry weight equivalent) of soil sample was suspended in 10 mL of sterile distilled water. Then, 1 mL of the soil suspension was serially diluted (six-fold) to estimate the fungal population. Incubation of the plates took place at ± 28°C for 4-5 days.

Purification of isolated fungi and identification

The isolated fungi were purified by point-inoculating them on plates that contained a PDA medium. Repeated point inoculations purified the fungi. The purity of the isolated fungus was confirmed by microscopic examination of the culture at 400X magnification using a light microscope. After ensuring purity, the culture was subcultured on PDA slants, allowing it to grow for 5-7 days. The stock cultures were stored at 4˚C, the worked and stock cultures were maintained, and the worked cultures were transferred to fresh PDA slanted at regular intervals of 3 months. The isolated fungi were subcultured on PDA, allowing them to grow and sporulate. Based on their colony and morphological characteristics, the fungi were identified. As a mounting fluid, a lactophenol cotton blue stain was used. The slides were observed under a microscope, and the fungi were identified using the mycological literature.

Production of protease

Submerged fermentation (SmF)

The 3% spore suspension (106 spores/mL) prepared from the PDA slant was inoculated in 200 mL of nutrient broth (NB) with a pH of 7 and then incubated in the flask at 35˚C 120 hours on a rotary shaker at a speed of 150 rpm. Then, the NB culture was centrifuged at 10,000 rpm for 15 minutes, and the supernatant thus obtained was used as crude enzyme extract. Then protease activity in the supernatant was detected.

Determination of proteolytic activity

The protease activity was tested using skim milk agar. For this, 0.01 mL (using a graduated micropipette) of crude enzyme produced from a local isolate of *Penicillium sp.* was added very carefully inside the wells in the center of the plates so as not to overflow the wells. Then the plates were incubated at 30˚C for 24 hours. The proteolytic activity was detected by observing the presence of clear zones. After initial screening for proteolytic activity, the *Penicillium sp.* strain exhibited activity for proteases and was used throughout the experiment.

Enzyme assay

At 50˚C, a reaction mixture containing 0.1 mL of an enzyme and three mL of 0.5% casein in 2.95 mL of 0.1 M Tris-HCl buffer (pH 8.5) was incubated. After 10 minutes, the reaction was stopped by adding 3 mL of cold 10% trichloroacetic acid (TCA). After 1 hour, the culture filtrate was centrifuged at 8,000 rpm for 5 minutes to remove the residue, and the absorbance of the supernatant was read spectrophotometrically at 280 nm. The enzyme activity was calculated by measuring the unit (mg) of tyrosine equivalent released and comparing it with the standard.

Effect of pH on protease production

The effect of pH on the protease activity produced by *Penicillium* was carried out using different pH ranges like 3, 4, 5, 6, 7, 8, 9, and 10.

Effect of temperature on protease production

Several temperature ranges, including 30˚C, 35˚C, 40˚C, 45˚C, 50˚C, and 55˚C, were used to study the impact of temperature on the activity of protease produced by *Penicillium sp.*

Molecular identification of fungus

The fungus samples were identified using 18s RNA sequencing from B.R. Biochem Life Sciences (New Delhi).

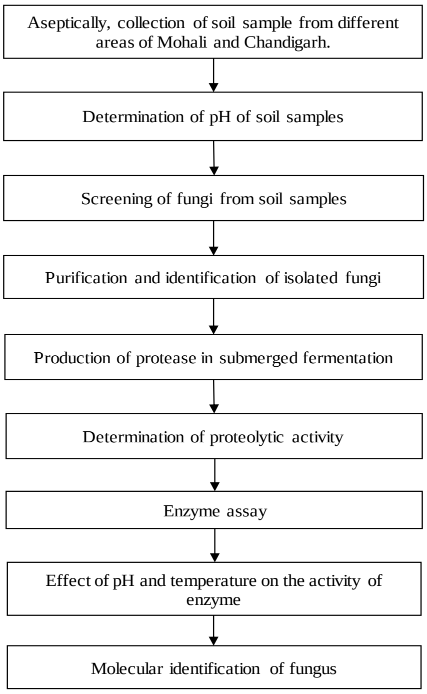


Figure 4 Flow chart depicting the study design

Results

In the present study, a total of 5 fungal forms were obtained from the positive samples. These included 1 fungal form from garden soils, 2 from crop fields, and 2 from lake soils (Error!

Reference source not found.). The soil samples from the crop field yielded the maximum number of fungi.

Table 11 Distribution of positive samples in different areas

Habitats	Place of sample collection	No. of samples	No. of positive samples	No. of fungi isolated
Garden soil	Chandigarh	3	2	1
Crop field (Rice)	Mohali	3	2	2
Crop field (Spinach)	Mohali	2	-	-
Lake soil	Sukhna lake	2	1	2

The data in Error! Reference source not found. indicated the dominance of Genus *Penicillium* in the soils of the surveyed habitats. Members of *Penicillium* have been widely reported in the soil since their nature allows them to have nutrients and moisture for all different life cycle studies.

Table 12 Percentage frequency of occurrence of fungal isolates

Fungal species	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Percentage frequency
Penicillium brevicompactum	+	+	+	-	-	-	50%
Penicillium islandicum	-	-	+	+	-	-	33.3%
Alternaria solani	-	+	-	-	+	+	50%

Effect of temperature

The medium was incubated at various temperatures (20-55°C). *Penicillium* showed maximal protease production at 35°C (Error! Reference source not found.). A significant range of proteases was produced at temperatures ranging from 30-55°C. Temperatures below 30°C, as well as above 55°C, harm enzyme production.

Effect of pH

Penicillium showed maximal protease production at pH 7, although significant levels of protease could be recorded at other pHs (**Figure 6**). The strain grew in the pH range of 3.0 to 9.0, with better protease yield. The pH values below 3.0 and above 9.0 harmed enzyme production.

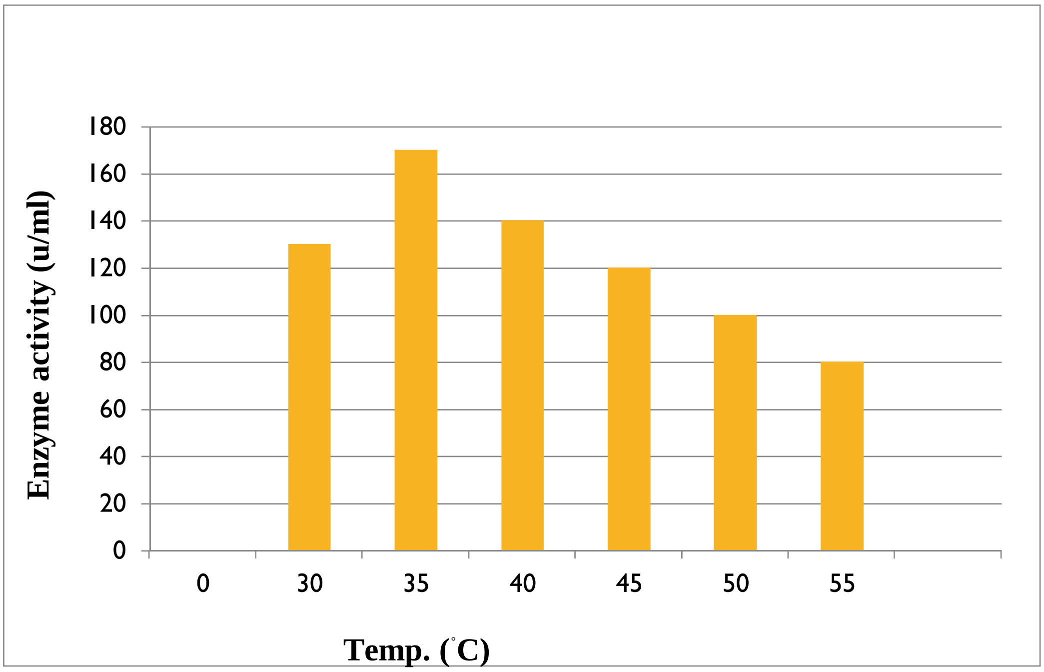


Figure 5 **Effect of temperature on enzyme activity**

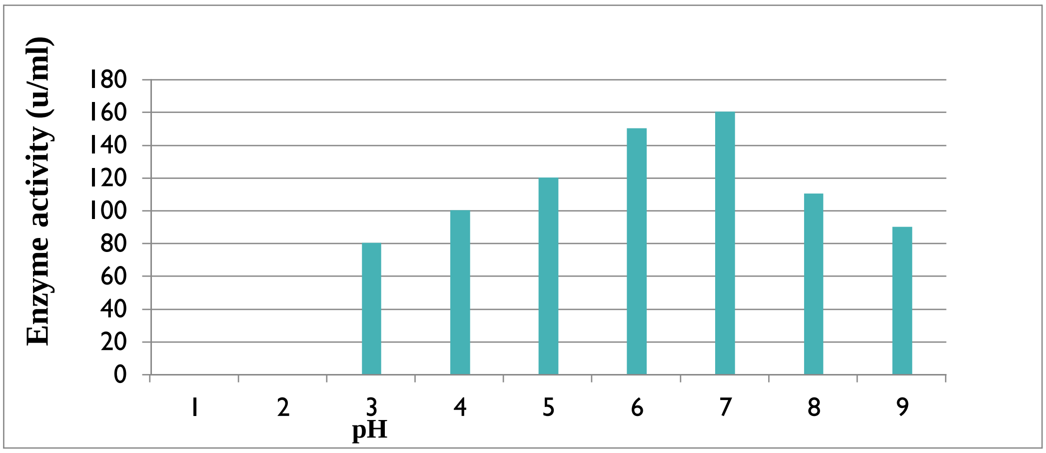
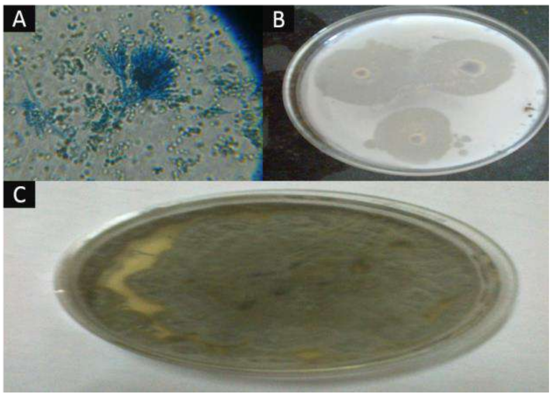


Figure 6 **Effect of pH on enzyme activity**

Effect of incubation time

The SmF medium was inoculated with the fungal strain and incubated at various time intervals (120 hrs). Error! Reference source not found. shows the effect of incubation time on protease production. *Penicillium* produced the highest amount of enzyme on the fifth day of



incubation.

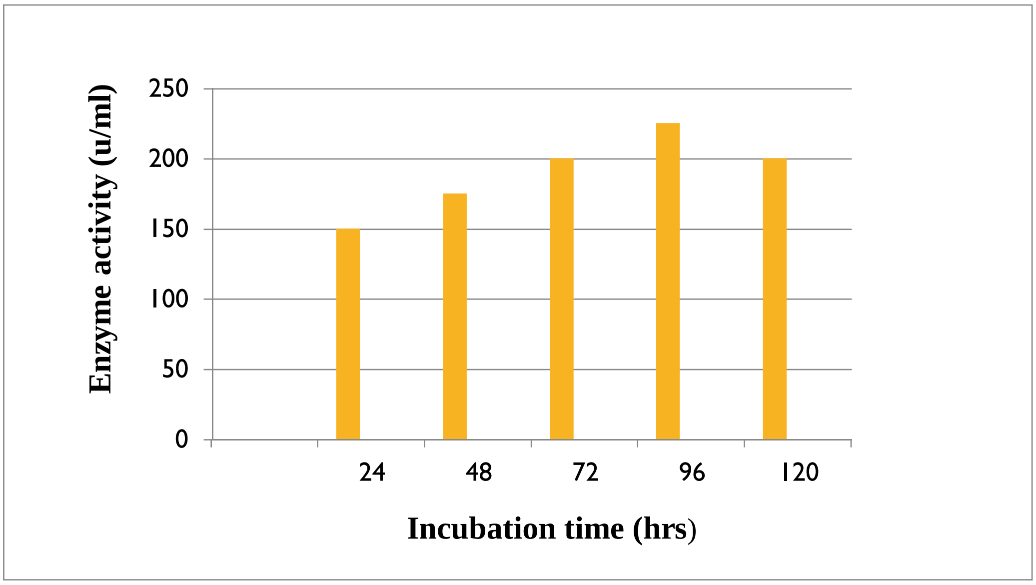


Figure 7 Effect of incubation time on enzyme activity

Figure 8 Microscopic view of a structure of A)* Penicillium sp.*, B) zone of inhibition shown on the skimmed milk agar plate, and C) pure culture of* Penicillium sp. *on PDA plates

Discussion

Fungi occurring in natural habitats with changing environmental conditions are important from an industrial point of view. These microorganisms are known to produce new metabolites or enzymes with enhanced catalytic characteristics. Therefore, an effort has been made in the current work to isolate the proteolytic fungi from soils obtained from various habitats. Twelve soil samples collected from different areas have been used to isolate fungi. These include 3 samples of garden soil, 5 crop fields, and 4 samples of lake soil from Punjab and Chandigarh. Out of these, a total of 9 samples were found to be positive for the occurrence of fungi.

The percentage frequency of positive samples in different areas was 50%, 33.3%, and 50% in samples collected from crop fields, garden soil, and lake soil. In the present study, a total of 3 fungal forms that produce the protease enzyme were obtained. These were identified and found to belong to *Alternaria* and *Penicillium sp.*

In the present study, 5 fungal forms were obtained from the positive samples. These include 1 fungal form from garden soils, 2 from crop fields, and 2 from lake soils. Soil samples from a crop field yielded the maximum number of fungi. These were identified and found to belong to 2 species of a single genus, including *Penicillium brevicompactum* and *Penicillium islandicum*. In the present study, the samples belonging to garden soil yielded only one fungal species, *Alternaria solani*, and *Cladosporium sp.* were isolated from lake soil samples. A total of two Penicillium *sp.* fungus forms have been isolated from Mohali samples.

The data indicated the dominance of the genus *Penicillium* in the soils of the surveyed habitats. Members of *Penicillium* have been widely reported in the soil since their nature allows them to have nutrients and moisture for all different life cycle studies.

Isolates of *Alternaria solani* have been recorded only in the soils of gardens. Gardens, crop fields, and other types of soil are regarded as rich environments for fungi with a variety of metabolic functions. In recent years, microbial proteases have replaced traditional proteases from animal and plant resources and have found various applications in recent eras. Proteases are widely distributed among microorganisms, including fungi, bacteria, and actinomycetes.[7] Proteases are well-known producers among fungi species such as *Aspergillus* and *Penicillium*[11, 14-16] and are reported to be active over a wide pH range.[5]

With at least 150 species, many of which have a similar shape, *Penicillium* is a wide genus. Many species also have great variability; at least 1,000 recognizably different phenotypes may eventually be cataloged. Even from common sources, only 70–80% of isolates are easily distinguishable because of the genus' inherent diversity. Many taxonomic keys to identifying *Penicillium sp.* are based primarily on morphological criteria. There are several classifications based on the micromorphology, macromorphology, and colors produced in the mycelium or diffused into the growing medium of the organism.[17] *Penicillium sp.* forms a well-developed septate mycelium with distinctive colors such as yellow, orange, red, or purple. The tips of unbranched conidiophores may terminate in a cluster of phialides or metulae. Each branch of branched conidiophores ends in a single or several metulae. The conidia are spherical to ellipsoidal and aseptate, and they may be blue-green, grey-green, or yellow, according to the species shown in Error! Reference source not found.**A**.

Protease activity was tested using skimmed milk agar. For this, 0.01 mL of crude enzyme made from a local strain of *Penicillium sp.* was put within the wells (by employing a graduated micropipette). The proteolytic activity was detected by observing the presence of clear zones. The zones of inhibition of 24 mm and 25 mm were formed due to the hydrolysis of milk proteins, as shown in **Figure 4**.

Conclusion

The present study obtained an extracellular protease from *Penicillium* and *Alternaria sp.* Instead of *Alternaria*, an isolated strain of *Penicillium sp.* was discovered to be a possible producer of extracellular protease. The enzyme yield was greater in *Penicillium sp.* than in *Alternaria sp.*
As a result of this fungus's high productivity in manufacturing the extracellular protease SmF, the data presented here point to the significance of a local isolate of *Penicillium sp.* Also, this study indicates the importance of the SmF technique since it is very simple in application and yields high-yield products. This strategy might be considered for large-scale applications in the production of industrial enzymes. Penicillium *sp.* growth in SF for large-scale protease production can be experimentally optimized using the study's large-scale protease production as a reference point.

Conflict of interest

The authors whose names are listed in the paper have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the paper's subject matter or accoutrements.

Data availability statement

The data can be made available upon request from the author.

Ethics statement

Not applicable.

Acknowledgement
Not applicable.

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Evaluation of Different Components of *Cannabis Sativa* and *Murraya Koenigii* for Antioxidant and Antibacterial Activity

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Abstract

Purpose: Herbal plants are rich in tannins, alkaloids, flavonoids, and phenolic compounds. Antioxidant supplements or meals containing antioxidants may help the body's defense system. Medicinal plants also inhibit the growth of different microorganisms like bacteria, fungi, viruses, etc. This study is exploring similar components in commonly available local plants, such as *Cannabis sativa* and *Murraya koenigii*.

Methods: An organic solvent was used to make a medical plant leaf extract (Methanol). The ability to reduce ferric ions was measured by the ferric reducing antioxidant power assay (FRAP), and the agar-well diffusion method was used to test the antibacterial activity of methanolic plant extracts. Plant extracts' antibacterial effectiveness is determined by the type and amount of phenolic content in the tissue.

Results: Analysis of *Murraya koenigii* leaves showed high levels of antioxidants such as phenolics and flavonoids but an absence of tannins, saponins, and volatile oils. *Cannabis sativa* samples showed positive levels of alkaloids, flavonoids, terpenes, steroids, and resins. The leaf extract was subjected to a phytochemical examination to evaluate the presence of phenol, flavonoids, tannins, alkaloids, saponins, and terpenoids. Plant extracts' antibacterial effectiveness is determined by the type and amount of phenolic content in the tissue.

Conclusion: *Murraya koenigii* and *Cannabis sativa* extracts have shown high antioxidant activity. Both plants' extracts have antibacterial action against a variety of microorganisms. The findings highlight the importance of scientific research to support ayurveda in the development of future drugs.

Keywords: *Murraya koenigii, Cannabis sativa,* antioxidant, antibacterial, microorganisms.

Introduction

Evaluation of new therapeutic products is a time-consuming and difficult process, but medicinal plants may be a feasible option because they have been studied by different ethnic groups since ancient times. For nearly 85% of the world's population, medicinal plants are regarded as the primary source of healthcare.[1]

Several synthetic medications on the market are produced from natural compounds derived from plants and microbes.[2] Herbal plants are rich in tannins, alkaloids, flavonoids, and phenolic compounds. These can be used to treat a wide range of medical conditions.[3,4]

The human body is extensively exposed to external sources of free radicals in today's world. As a result, the body's antioxidative defense system may not be sufficient to fully avoid oxidative damage. Antioxidant supplements or meals containing antioxidants may help the body's defense system and contribute to the reduction of oxidative damage in this way.[5]

Medicinal plants also inhibit the growth of different microorganisms like bacteria, fungi, viruses, etc. The antibacterial properties of plants like *P. erinaceus* and B. salicina are well established.[6,7] The most famous use of medicinal plants for the cure of infectious diseases is the discovery of artemisinin from Artemisia annua for the treatment of malaria.[8] Plant extracts from *Andrographis paniculata, Chrysopogon zizanoides, Cyperus rotundus, and Mollugo cerviana* have also shown activity against coronaviruses.[9] As a result, a number of investigators in Asia and Africa have investigated the activities of plant extracts, fractions, and pure chemicals present in the extracts on a variety of microorganisms related to bacterial and fungal illnesses.[10-12]

This study is also exploring similar components in commonly available local plants, such as *Cannabis sativa* and *Murraya koenigii*, in order to meet rising healthcare demands in a cost-effective manner.

Methods

Extract preparation

Herbal plant leaves (*Murraya koenigii* and *Cannabis sativa*) were collected in sterile containers. Medicinal plant leaves were cleaned, measured, and washed many times with water to eliminate dirt before being air-dried in a shed at room temperature (26˚C) for two weeks. Then, using an electronic grinder or mortar and pestle, it was crushed to a homogenous powder and stored in sterile containers for later use. An organic solvent was used to make a medical plant leaf extract (Methanol). For 2-3 days, the dry plant powder was soaked in methanol with intermittent shaking. It was filtered via Whatman filter paper at the end of the extraction procedure. This methanolic filtrate was concentrated at 40˚C on a rotary evaporator under reduced pressure and then stored at 4˚C. This methanolic filtrate was concentrated at 400˚C on a rotary evaporator under reduced pressure and then stored at 40˚C for later use. To get a known concentration of methanol extract, the filtrate was reconstituted in a known amount of dimethylsulfoxide (DMSO).

Phytochemical analysis

Cannabis sativa

The leaf extract was subjected to a phytochemical examination to evaluate the presence of flavonoids, tannins, phenols, saponins, terpenes, steroids, resins, and volatile oils. Handpicked 500g of leaves of C. sativa were separated from stems and twigs and then crushed using an electronic grinder. 5g of dried powdered leaves were soaked in 200 ml of petroleum ether for 24 hours at 25˚C to make a stock solution of C. sativa. After evaporating the solvent at room temperature (26˚C), 25 ml of acetone was used to dissolve the resin, yielding a solution with a concentration of 12 mg/ml.

Murraya koenigii

Murraya koenigii L. leaves were completely washed 2-3 times with running tap water and once with sterile water, air dried for 6-7 days in the shade, then mechanically pulverized using a wooden mortar and pestle. The pulverized plant material was stored in airtight containers to keep it dry. The leaf extract was subjected to a phytochemical examination to evaluate the presence of phenol, flavonoids, tannins, alkaloids, saponins, and terpenoids.

Antioxidant activity

DPPH assay

The sample's radical scavenging potential was measured by measuring the drop in absorbance caused by DPPH at 517nm, which represents the creation of its reduced form, 2,2-diphenyl-1-picrylhydrazine (DPPH), which is yellow in color. The purple-colored methanolic solution shows high absorption at 517 mµ due to the presence of odd electrons in the sample.[13]

FRAP assay

The ability to reduce ferric ions was measured by the ferric-reducing antioxidant power assay (FRAP). The flavonoids and phenolic acids present in the medicinal plant exhibit strong antioxidant activity, which is dependent on their potential to form complexes with metal atoms, particularly iron and copper. This method is based on the principle of increasing the absorbance of the reaction mixtures. The antioxidant activity rises in tandem with the absorption. The antioxidant compound present in the samples forms a colored complex with potassium ferricyanide, trichloroacetic acid, and ferric chloride, which is measured at 700 nm by an UV spectrophotometer according to the color of the reaction mixture.14

Antibacterial activity

The agar-well diffusion method was used to test the antibacterial activity of methanolic plant extracts. The agar-well diffusion method is commonly used to test plant extracts for antibacterial activity. The antagonistic activity of the extracts against different pathogenic bacteria was tested using the agar-well diffusion method. Plant extracts' antibacterial effectiveness is determined by the type and amount of phenolic content in the plant tissue, as well as the infection's innate resistance. In millimeters, the diameter of the created zone of inhibition can be measured.

Statistical analysis

Concentration and absorbance calculations were done by placing the constant values of m and c in the equation.

$Y = mx + c$

Where x = Conc. (µl/ml), m = Slope, and c = Intercept

Where one value is given, the other can be calculated; i.e., if the values of absorbance are given, then concentration values can be calculated from the above formula by placing the values of m and c as constant values.

The following equation was used to compute the percentage inhibition used to indicate the capacity to scavenge the DPPH radical:

DPPH scavenging activity (%) = $(A_0 - A_1)/A_0 \times 100$

Where A_0 denotes the control's absorbance and A_1 denotes the sample's absorbance.

Results

Phytochemical analysis

Phytochemical analysis of Cannabis sativa samples showed that the plant was rich in alkaloids, flavonoids, terpenes, steroids, and resins, showing positive results, whereas results for tannins, saponins, and volatile oils were negative, confirming the absence of these particular groups.

On the other hand, phytochemical analysis of Murraya koenigii samples showed that the plant was rich in phenol, flavonoids, tannins, alkaloids, and saponins, showing positive results. As shown in Table 13 and Table 14.

Table 13 Results of phytochemical screening of the extract of Cannabis sativa

Extract constituents	**Tests**	**Results**
Alkaloids	Dragendroff`s	Positive
Saponins		Negative
Terpenes & Steroids	Burchard	Positive
Tannins		Negative
Flavonoids	Lead acetate	Positive
Resins		Positive
Volatile oils		Negative

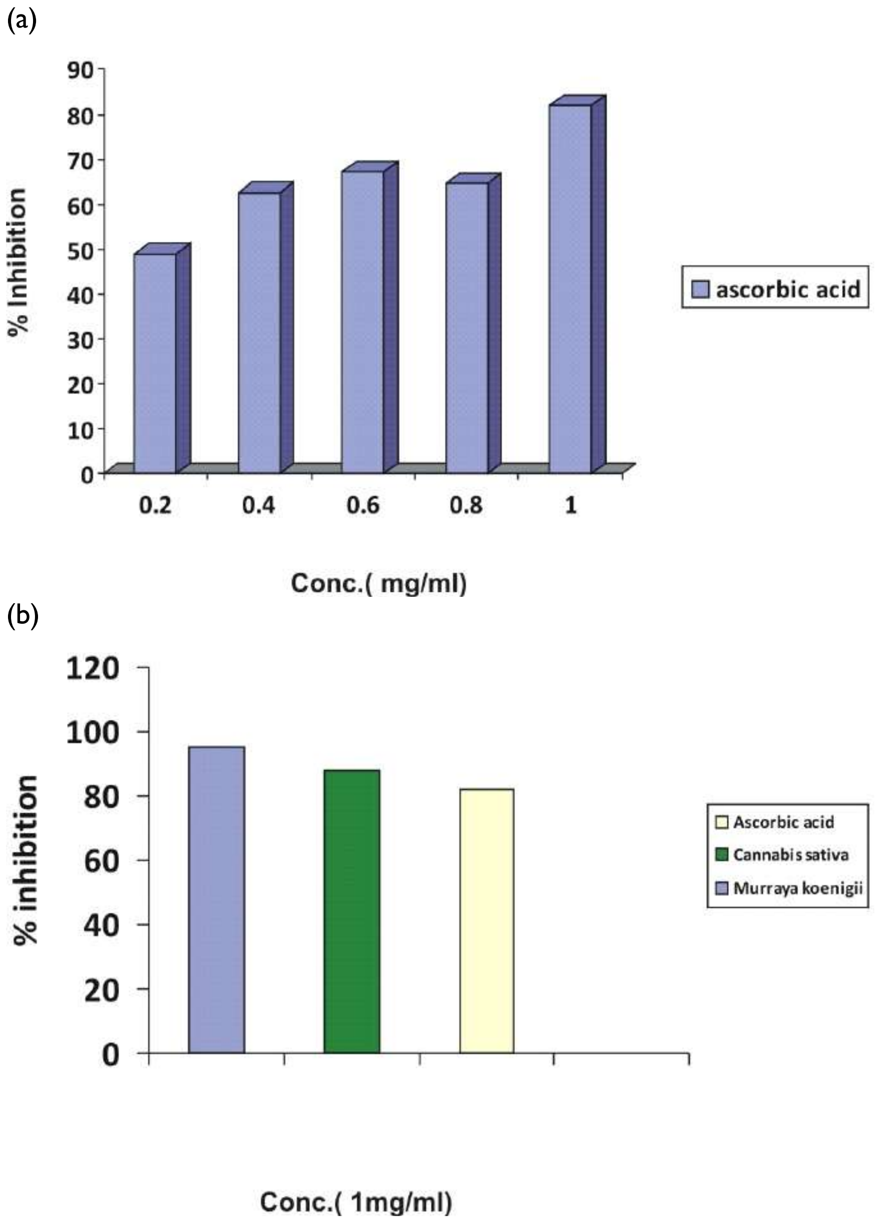
Table 14 Results of phytochemical screening of the extract of Murraya koenigii

Extract constituents	**Results**
Phenol	Positive
Tannins	Positive
Saponins	Positive
Alkaloids	Positive
Flavonoids	Positive

Antioxidant activity

In this activity, the extract was measured by a DPPH radical scavenging assay. The results of this assay are plotted on graphs (Figure 9). The DPPH assay has a greater ability to scavenge free radicals, which shows more antioxidant activity. It was observed that with the increasing concentration of extract, the free radical scavenging activities also increased. In the present study, we observed that extract of Murraya koenigii leaves showed high inhibition as compared to Cannabis sativa in the case of DPPH radical scavenging activity, i.e., 95.32%, whereas Cannabis sativa showed low inhibition, i.e., 88.10%.

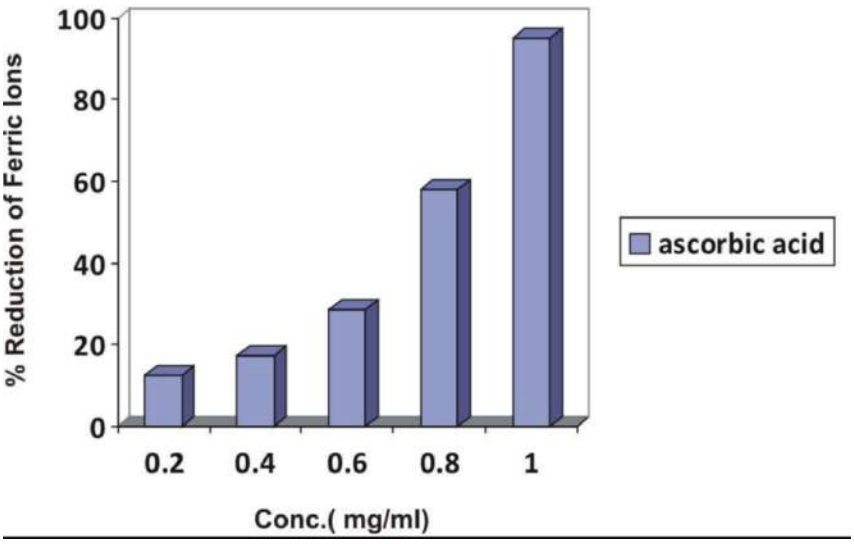
Figure 9 (a) DPPH free radical scavenging activity of ascorbic acid (b) DPPH free radical scavenging activity (1mg/1ml)



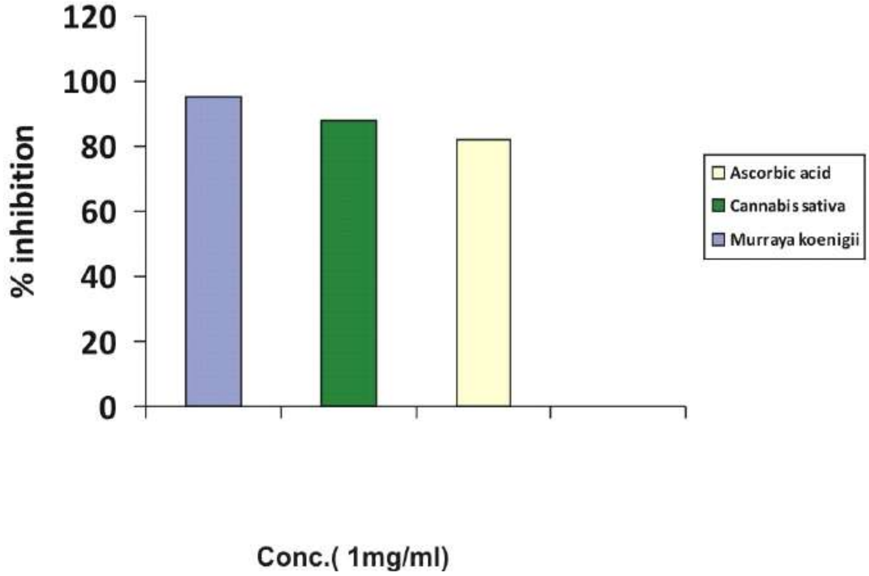
A Fe3+ to Fe2+ reduction assay was used to test the extracts' and fractions' lowering capacities. Depending on the content of antioxidants in the samples, the yellow hue changes to a pale green or blue tone. *Murraya koenigii* and *Cannabis sativa* extracts also had high levels of antioxidants such as phenolics and flavonoids. All of the samples demonstrated reducing capacity in a concentration-dependent manner, as assessed by a colorimeter at 520nm and displayed on graphs (**Figure 10**).

Figure 10 **(a) FRAP: Ferric ions reducing capacity (%) of ascorbic acid (b) FRAP: Ferric ions reducing capacity (%)**

(a)



(b)



A FRAP assay (ferric reducing antioxidant power assay) was also performed, which measures an antioxidant's reducing capacity. In this investigation, we discovered that *Murraya koenigii* extracts have a higher percent reduction of ferric ions (92.28%) than *Cannabis sativa* extracts (83.24%).

Antibacterial activity

As shown in **Table 15** and **Table 16**, the agar-well diffusion method was used to assess antibacterial activity. *Cannabis sativa* and *Murraya koenigii* methanolic extracts showed antibacterial action against a variety of stains *(E. coli*, *Salmonella typhimurium*, and *Staphylococcus pyogenes*). The highest activity, or zone of inhibition, was seen against *E. coli* (23 mm) and the lowest against *Staphylococcus pyogenes* (11 mm) in the case of

Murraya koenigii leaf extract. In the case of *Cannabis sativa* leaf extract, the highest activity or zone of inhibition is observed against *Salmonella typhimurium* (22 mm), and the lowest activity or zone of inhibition is observed against *E. coli* (12 mm). As a control, DMSO is utilized, which shows no inhibitory zone.

Table 15 Antimicrobial activity of Murraya koenigii

Bacterial Strains	**Control**	**20 µl**	**40 µl**	**60 µl**	**80 µl**	**100 µl**	**200µl**
Zone of inhibition (mm)							
Staphylococcus pyogenes	0	13	17	19	20	23	0
E.coli	0	11	14	16	17	19	0

Table 16 Antimicrobial activity of *Cannabis sativa*

Bacterial Strains	**Control**	**20µl**	**30 µl**	**40 µl**	**50 µl**	**60 µl**
Zone of inhibition (mm)						
Staphylococcus pyogenes	0	12	14	17	20	21
E.coli	0	15	16	17	19	22

Discussion

The extract of *Murraya koenigii* leaves had a higher percentage inhibition of DPPH radical scavenging activity than *Cannabis sativa*, i.e., 95.32% compared to 83.24%, but these results show high antioxidant activity. Also, curry (*Murraya koenigii)* leaf extract showed a higher percentage reduction of ferric ions (FRAP assay).

The extracts from both plants also showed antibacterial activity against different stains (*E. coli, Salmonella typhimurium,* and *Staphylococcus pyogenes*).

The phytochemical examination of *Murraya koenigii* revealed the presence of alkaloids, flavonoids, phenols, saponins, and tannins in this study. *Murraya koenigii* is an anti-diabetic.[15] Saponin aids in the reduction of cholesterol and blood pressure. Many other review articles have highlighted the importance and phytochemical activity of *Murraya koenigii*. Many other studies have found medicinal effects from the essential oil prepared from *Murraya koenigii*.[16, 17] Many of these phytochemical products are already in non-clinical trials against different diseases, but more research is still needed to isolate more such components to be used in other healthcare spheres.[18, 19]

The leaf extract was subjected to a phytochemical examination to evaluate the presence of flavonoids, tannins, phenols, saponins, terpenes, steroids, resins, and volatile oils in the leaves of *Cannabis sativa*. Due to their physiological effects, such as their capacity to scavenge free radicals and possess anti-mutagenic and anti-inflammatory properties, these phenolic classes have drawn a lot of interest.[20, 21] Flavonoids, which have strong antioxidant properties, are the most prevalent phenolic chemical families found in plants.[22] Flavonoids are naturally occurring in plants and have positive effects on human health. Studies on flavonoidic derivatives have shown a wide range of anti-bacterial, anti-viral, anti-cancer, anti-mutagenic, anti-inflammatory, and anti-allergic activities.[23, 24] The results show that the extract of cannabis shows the presence of alkaloids, flavonoids, etc. Thus, extracts possessing good free radical scavenging activity would be promising for the development of functional foods and nutraceuticals, for example, with cancer-preventing properties. Including this study, many other studies have shown these results.[25]

These findings emphasize the relevance of conducting scientific studies to support ayurveda or other traditionally made medications in the development of future drug discoveries.

Conclusion

Murraya koenigii and *Cannabis sativa* extracts have shown high antioxidant activity. Both plants' extracts have antibacterial action against a variety of microorganisms. The findings highlight the importance of scientific research to support ayurveda in the development of future drugs.

Conflict of interest

The authors whose names are listed in the paper have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers’ bureaus, membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the paper's subject matter or accoutrements.

Data availability statement

The data can be made available upon request from the author.

Ethics statement

Not applicable.

Acknowledgement

Not applicable.

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Assessment of Adherence to Guidelines in the Treatment of Patients with Type 2 Diabetes Mellitus at Selected Clinics in Punjab: A Retrospective Observational Study

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Abstract

Purpose: To identify the drug prescription patterns in patients with type 2 diabetes mellitus (DM). Other objectives were to analyze demographic data and HbA1c levels of patients and assess their pharmacological therapy plans.

Methods: A retrospective observational study was conducted at diabetes clinics in the Punjab region of India. The subjects who fulfilled the inclusion criteria were enrolled in the study. The collected data were analyzed with the help of SPSS ver. 24.

Results: As per the disposition data, the number of female patients with DM was greater than the male patients. Type 2 DM was found to be common among the patients over the age of 60 years and 54 patients have had the disease for 5-10 years. A majority of the patients enrolled in the study had normal renal functions. A significant relationship was found between HbA1c levels and age ($p<0.001$) and disease duration ($p<0.005$). Evaluation report of the drug prescription pattern revealed that the long-acting insulin analogs were widely prescribed to the patients along with metformin.

Conclusion: Any standard prescribing pattern was not found which was advised by the national or international standard guidelines. This can be attributed to the population size, different prescribers, and inter-individual variation and may be due to some factors not found in the patient’s medical record. Regardless of the cause, there has to be adherence to the most recent standard guidelines that are supported with evidence to improve the health of patients. With regard to the International Diabetes Federation (IDF) guidelines, there was no adherence to these sets of standard guidelines in most of the prescription patters for patients.

Keywords: Type 2 diabetes mellitus, Prescription pattern, International diabetes federation guidelines, Medication adherence.

Introduction

Type 2 diabetes mellitus (DM), also known as insulin non-dependent diabetes mellitus (INDDM), affects the individual in an old age, obesity, and insulin resistance. If left untreated, type 2 DM may lead to nephropathy, neuropathy, retinopathy, and cardiovascular complications.[1-3] Type 2 DM constitutes about 85% to 95% of the population with diabetes in developed countries and even higher in the developing countries. In 2003, 194 million people (aged 20 to 79 years) had diabetes and a quarter of them belonged to the developing countries. There is a rapid increase in the prevalence of diabetes in the Asian countries.[4] Diabetes, if not well controlled, may cause blindness, kidney failure, lower limb amputation, and several other long-term consequences that impact significantly on the quality of life.[5,6] Where data are available – mostly from high-income countries – prevalence, incidence, and trends vary hugely between countries.[7] In 2010, diabetic retinopathy caused 1.9% of moderate or severe visual impairment globally and 2.6% of blindness.[6,8] Pharmacological approach involves medication therapy while the non-pharmacological approach involves reduction of weight, alcohol intake, and smoking, and increase in exercise.[9] Proper use of the drug has an enormous contribution to the reduction of death and diseases all over the world due to its subsequent checkup, and public and economical reimbursement.[10] Irrational use of drugs will lead to failure of treatment, resistance, and toxicity of drug as well as affect the quality of drug therapy.[11] One-half of all the drugs that are prescribed, dispensed, and sold worldwide are inappropriate, while half of the patients fail to take the drugs properly and 1/3rd of them fail to access to the essential drug list. This is why it is necessary to introduce drug use patterns and focus on the irrational prescribing pattern.[12] The pattern of drug use is different in different countries. Problems related to drug use may be due to practitioner, distributer, and consumer and may be due to health care facility that further worsens the medication management.[13] Thus, the overall aim of this study was to explore the current status of the adherence of anti-diabetic drugs to the international guidelines (as prescribed by the international federation) by medical practitioners and the current status of prescription.

Methodology

A retrospective observational study was conducted at selected clinics in the Malwa region of Punjab, India. A total of 103 patients diagnosed with type 2 DM were enrolled in the study by considering the inclusion criteria of both genders and signing the informed consent form. The investigation was carried out for 6 months after obtaining approval from the Institutional Ethics Committee (IEC) and the data of consecutive 5 years were collected from the Medical Record Department (MRD) with the help of a data collection form. Steps required for the evaluation of the prescription are mentioned in **Figure 1**.

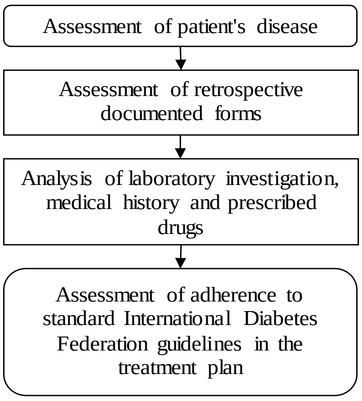

Figure 1 Evaluation of Prescriptions

Data Analysis

The collected data was analyzed via SPSS Ver. 24. Descriptive statistics were used to analyze various parameters taken in the study.

Results

Demographic and Baseline Characteristics

An aggregate of 103 cases were enrolled in the study. The number of female patients with type 2 DM was higher than the male patients (56 [57.2%] vs 44 [42.7%]). The highest rate of type 2 DM was found to be among patients >60 years of age (41 [39.8%]), followed by patients between the age of 50-59 years (32 [31.0%]; **Table 1**).

Table 1 Demographic and Baseline Characteristics

Characteristic Statistic	**N=103**
Age	
Mean ± SD (years)	56.02 ± 10.944
Age distribution, n (%)	
20-29 yrs	2 (1.9)
30-39 yrs	6 (5.8)
40-49 yrs	22 (21.3)
50-59 yrs	32 (31.0)
>60 yrs	41 (39.8)
Gender, n (%)	
Male	44 (42.7)
Female	59 (57.2)
BMI, (kg/m^2)	
Median	26.47
Interquartile range	8.62
BMI distribution	
<18.5 kg/m^2 (underweight)	2
18.5-25 kg/m^2 (normal)	28
25-30 kg/m^2 (overweight)	47
>30 kg/m^2 (obese)	26
Disease duration, n (%)	
<2 yrs	1 (0.9)
2-5 yrs	18 (17.4)
5-10 yrs	54 (52.4)
>10 yrs	30 (29.1)
eGFR for Renal function, n (%)	
>90 mL/min/1.73 m^2 normal kidney function	97 (94.17)
89-60 mL/min/1.73 m^2 mild kidney damage	1 (0.97)
59-30 mL/min/1.73 m^2 mild to moderate kidney damage	4 (3.88)
29-15 mL/min/1.73 m^2 severe kidney damage	1 (0.97)
<15 mL/min/1.73 m^2 severe kidney damage	0 (0)
HbA1c, n (%)	
6-8%	6 (5.8)
8-10%	12 (11.6)

Characteristic Statistic	N=103
10-12%	74 (71.8)
12-14%	11 (10.6)
Age distribution of HbA1c, n (%)	
6-8%	
20-29 yrs	2 (33.3)
30-39 yrs	1 (16.7)
40-49 yrs	1 (16.7)
50-59 yrs	1 (16.7)
>60 yrs	1 (16.7)
8-10%	
20-29 yrs	0 (0)
30-39 yrs	3 (25)
40-49 yrs	4 (33.3)
50-59 yrs	3 (25)
>60 yrs	2 (16.7)
10-12%	
20-29 yrs	0 (0)
30-39 yrs	2 (2.7)
40-49 yrs	17 (23)
50-59 yrs	26 (35.1)
>60 yrs	29 (39.2)
12-14%	
20-29 yrs	0 (0)
30-39 yrs	0 (0)
40-49 yrs	0 (0)
50-59 yrs	2 (18.2)
>60 yrs	9 (81.8)
Route of administration of anti-diabetic drug, n (%)	
Tablet (Oral)	40 (38.8)
Injection	8 (7.7)
Tablet + Injection	55 (53.3)

N = No. of subjects; BMI = body mass index; SD = Standard deviation.

Body Mass Index (BMI)

Upon evaluation of the patient’s body mass index (BMI), patients with a BMI of 25-30 kg/m^2 had the highest prevalence of DM with 19.4% and 26.2% for male and female, respectively.

Disease Duration

Over 30 (29.1%) patients have had the disease for more than 10 years, while 54 (52.4%) patients have had it for 5-10 years.

Renal Function

A majority of the patients (97 [94.17%]) had normal kidney function, while 1 (0.97%) patient had severe kidney damage that required an adjusted therapy plan. Based on the outcomes of the study, renal functioning was significantly related to age ($p<0.005$).

HbA1c Test Evaluation

A total of 74 (71.8%) patients had high HbA1c levels (10-12%), and 11 (10.6%) had very high levels (12-14%; **Figure 2**). There was a significant relationship between HbA1c levels and age *(*p<0.001*)* and disease duration (p<0.005).

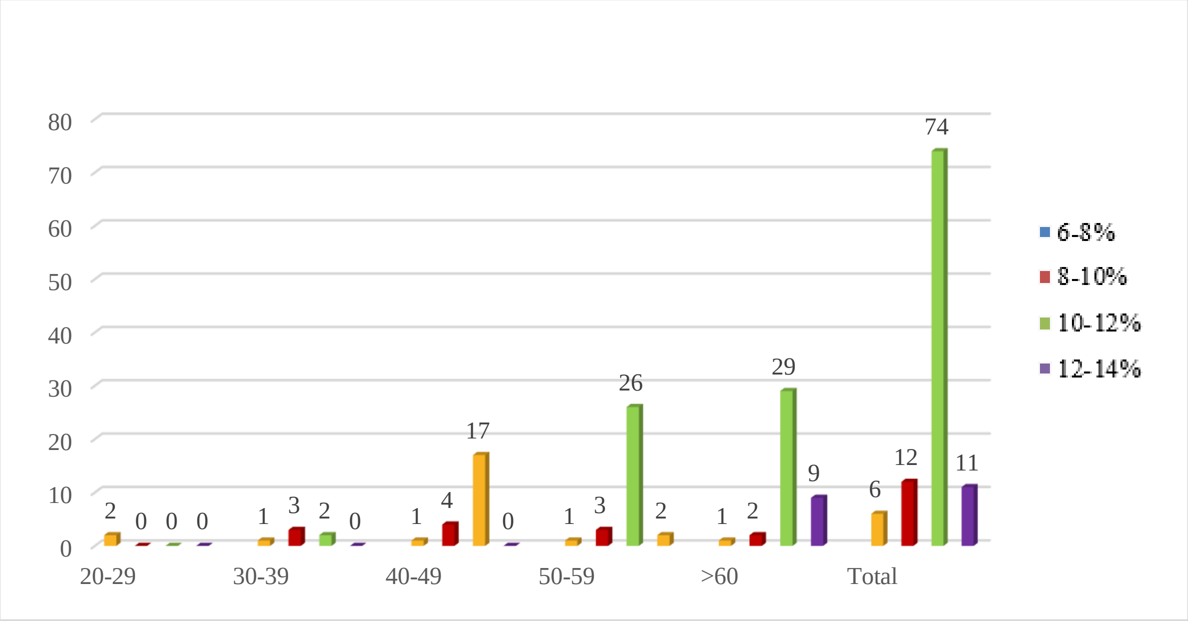

Figure 2 HbA1c levels

Evaluation of Route of Administration

Of 103 patients with anti-diabetic prescriptions, 40 (38.8%) patients were taking oral prescriptions (tablets), 8 (7.7%) patients were taking injections, and 55 (53.3%) patients were taking both oral prescriptions (tablets) and injections.

Drug Prescription Pattern in Patients with Type 2 DM

Most of the patients (40 [38.8%]) were prescribed long-acting insulin analog injections (insulin detemir and insulin glargine) and metformin tablets (**Table 2**), and 22 (21.3%) of the patients were prescribed a combination of metformin and sulfonylurea. Few patients had metformin only or sulfonylurea only prescriptions, comprising 2 (1.9%) and 3 (2.9%), respectively, while 13 (12.6%) patients had a prescription of metformin with rapid-acting insulin. Three-drug therapy or two drugs plus insulin were also observed that made up 15 (14.5%) of the total patients.

Table 2 **Drug Prescription Pattern**

Antidiabetic drugs used by patients	**n (%)**	**Glycated hemoglobin (HbA1c) test (%)**			
		6-8	**8-10**	**10-12**	**12-14**
Metformin (GC2)	2 (1.9)	2	0	0	0
Sulfonylurea (GC2)	3 (2.9)	1	2	0	0
Metformin + sulfonylurea (GC3)	22 (21.3)	3	10	9	0
Metformin + α-glucosidase inhibitor / DPP-4 inhibitor / thiazolidinedione (GC3)	0	0	0	0	0
Metformin + rapid-acting insulin secretagogue (GC3)	13 (12.6)	0	0	13	0
3-drug therapy or 2 drugs + insulin (GC4)	15 (14.5)	0	0	11	4
NPH insulin, insulin glargine or insulin	40 (38.8)	0	0	36	4

determir + metformin (GC5)					
Biphasic insulin + metformin (GC5)	8 (7.7)	0	0	5	3
Basal plus mealtime insulin regimen + metformin (GC5)	0	0	0	0	0

GC2 - First-line therapy; GC3 - Second-line therapy; GC4 - Third-line therapy; GC5 - Fourth-line therapy

Treatment Concerning Disease Duration

Based on the results of the study, antidiabetic agent treatment was significantly associated with the disease duration of the patient (p<0.005; **Table 3**).

Table 3 Treatment Concerning Disease Duration

Antidiabetic drugs used by patients	Disease duration (years)			
	<2	2-5	5-10	>10
Metformin	0	2	0	0
Sulfonylurea	0	3	0	0
Metformin + Sulfonylurea	1	7	14	0
Metformin + α-glucosidase inhibitor / DPP-4 inhibitor / thiazolidinedione	0	0	0	0
Metformin + rapid-acting insulin secretagogue	0	1	10	2
3-drug therapy or 2 drugs + insulin	0	0	3	12
NPH insulin, insulin glargine or insulin determir + metformin	0	5	23	12
Biphasic insulin + metformin	0	0	4	4
Basal plus mealtime insulin regimen + metformin	0	0	0	0

Treatment for Renal Function

Patients with moderate to low renal function constituted 4.85% of the total subjects under study and required special care for their treatment as per their renal condition. **Figure 3** depicts the treatment plan given to patients with renal disease.

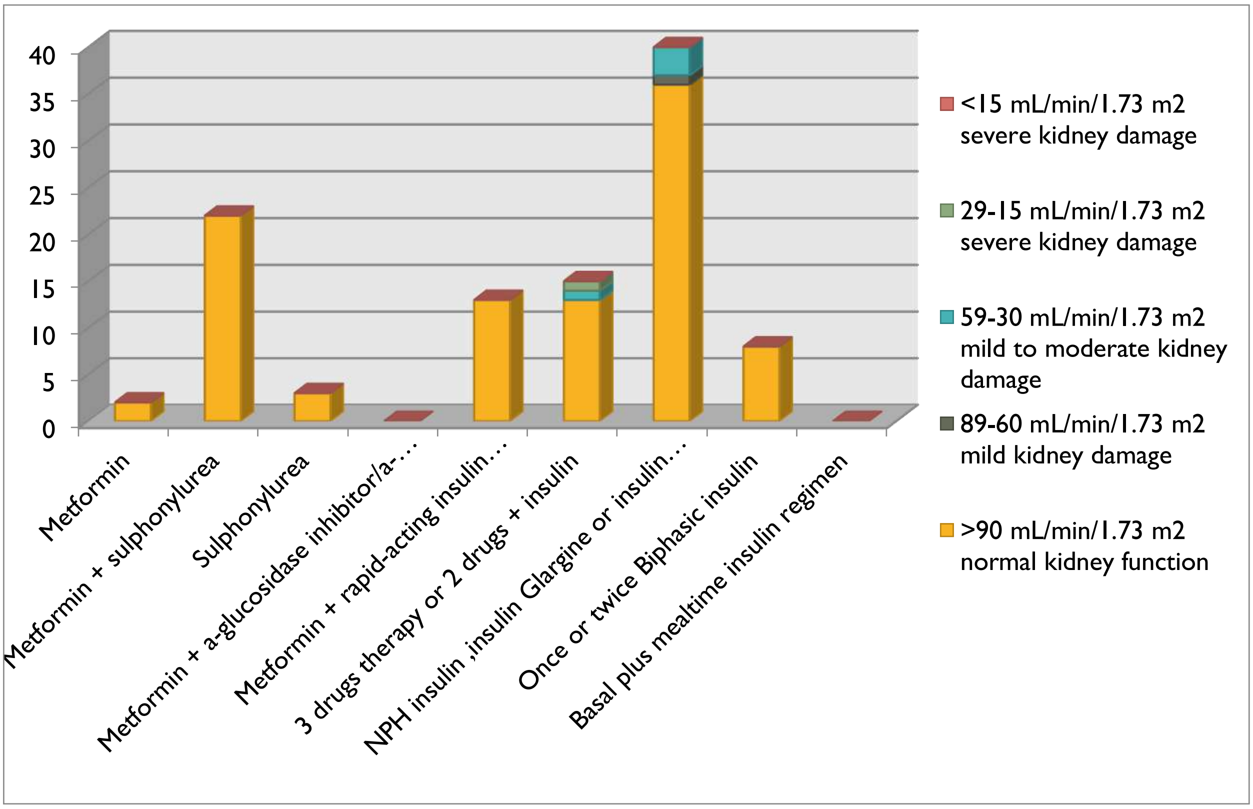

Figure 3 Drug therapy plan for patients with a renal condition

Glycated Hemoglobin vs. Disease Duration

Our investigation also reported that glycated hemoglobin was strongly related to disease duration ($p<0.005$). The following **Table 4** illustrates the effectiveness of the therapy over the years.

Table 4 Drug Response of Patients

HbA1c (%)	**Disease duration (years)**			
	<2	**2-5**	**5-10**	**>10**
6-8	1	5	0	0
8-10	0	4	8	0
10-12	0	8	44	22
12-14	0	1	2	8

Discussion

After collecting data from 103 patients, evaluating and analyzing their prescriptions and disease state, these results do not indicate the actual prevalence of diabetes among men and women, due to sample size restriction. With the highest BMI values belonging to women, 26.2% of the total population was women who were overweight (25-30 kg/m^2). Women with obesity (>30 kg/m^2) were also higher than to men (13.5% vs 11.6%). Nevertheless, previous studies show that men are more at risk of developing type 2 DM compared to women of the same age.[14] This was confirmed by evaluating the BMI of both genders. Men tend to develop the condition at lower BMI than women of the same age do.

As the body grows over time, there is a gradual change in physiology due to wear and tear that occurs over time. This makes the elderly population more susceptible to illnesses. For that reason, it is no surprise that the highest prevalence of type 2 DM is found in elderly population in this study (39.8% and 31.0% for patients over 60 and 50-59 age groups, respectively). Limited physical activity, co-morbidities, and patient compliance adversely affect the progression of the disease and treatment to a greater extent in the elderly population compared to the younger population.

Glycated hemoglobin (HbA1c) is one of the most important tests to indicate how well the disease is controlled.[15] It provides a long-term indicator for blood sugar levels. Many national and international guidelines recommend a target level of <7%. Analyzing the test results collected from patients, 94% patients had high HbA1c levels (≥8% to 14%), which indicate ineffective treatment, poor patient compliance to therapy, exercise, and diet recommendations.

According to patients' renal function, the prescribed treatment to the six patients which includes metformin, was not as per IDF guidelines as the mild and moderate kidney damage could be aggravated by metformin. However, the FDA announced a revised warning use of metformin in certain patients with reduced kidney function. They have concluded from the review of studies published in the medical literature that metformin can be used safely in patients with mild impairment in kidney function and some patients with moderate impairment in kidney function.[16] It was observed that 2 out of 6 patients with a relatively low HbA1c (6-8%) were prescribed metformin only therapy, which was not per IDF guidelines. Three metformin + sulfonylurea prescriptions were also observed and identified as irrational since this combination can cause hypoglycemia. One patient was receiving a sulfonylurea only treatment, which, according to guidelines, can be chosen as an alternative to metformin.

Second-line therapy of two anti-diabetic drugs (metformin + sulfonylurea, metformin + α-glucosidase inhibitor / DPP-4 inhibitor / thiazolidinedione, metformin + rapid-acting insulin secretagogue) was prescribed to 35 patients, while only 12 patients had HbA1c levels of 8-10%. We conclude that some patients were either overprescribed or under-prescribed. In both cases, it is considered as an irrational treatment that may lead to severe consequences to the patients. Third-line and fourth-line therapies as per the IDF guidelines include adding insulin to the treatment plans, which is justified in patients who have high levels of HbA1c to control their blood glucose levels. Prescribers use different strategies regarding insulin, and treatment should be individualized as per the patients' health state taking particular note of the cost and availability of generic drugs. However, 24 patients who have had high HbA1c levels and have not been prescribed any form of insulin, which can aggravate the disease complications. As per the recommended guidelines GC6, insulin therapy should not be unduly delayed.

Type 2 DM is known to be a progressive disease, and as such treatment should be adjusted according to the blood glucose levels. It was observed that the HbA1c levels were 10-12% high in patients who have had the disease for 5-10 years (44 patients) and over 10 years (22 patients), 12-14% in eight patients who have had the disease for more than 10 years. These numbers indicate inefficient treatments and/or poor patient compliance. The overall status of control of the disease is less than satisfactory.

Conclusion

After careful analysis of data, we conclude that the treatment practices for type 2 DM were not as per the IDF guidelines. Irrationality in treatment and disease management were detected which, can lead to decreased patient satisfaction, high therapy costs, aggravating the disease state, and possible failure of therapy. Many patients require rectification of therapy plans to control their rather high blood glucose levels regardless of current oral antidiabetic agents and put to insulin regimen as per the prescribed guidelines to avoid further complication. It must be noted that all studies of this kind must be considered as a feedback to the policy makers, physicians, hospital administrations, governments, organizations, and related parties in the health care system for future references and the betterment of patient's health.

Improper control of diabetes can put patients at a risk of advancement of long-term physiological and psychological complications. Therefore, it is of utmost importance to provide the best care to patients. This can be achieved only by following the evidence-supported standard guidelines in the diagnosis and treatment of diabetes to provide optimum health care and ensure maximum benefit utilization of available choices of drugs.

Regardless of the cause, there has to be adherence to the most recent standard guidelines which are supported with evidences to improve health care provided. With regard to the IDF guidelines specially, there was no adherence to these sets of guidelines in most patients.

Conflict of interest

The authors whose names are listed in the paper have no confederations with or involvement in any organization or entity with any financial interest (similar to honoraria, educational grants, participation in speakers' divisions, membership, employment, consultancies, stock ownership, or other equity interest; and expert evidence or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, confederations, knowledge, or beliefs) in the subject matter or accouterments bandied in this paper.

Data availability statement

The data can be made available upon request from the author.

Ethics statement

This study was approved by the ethics committee. Participants were informed at the beginning of the study that it was voluntary and that they could quit at any time. All study participants gave written informed consent.

Acknowledgement

Not applicable.

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