ISSN Print: 2518-4261
ISSN Online: 2518-427X
Vol. 61(2), June 2024

PROCEEDINGS
OF THE PAKISTAN ACADEMY OF SCIENCES:
B. Life and Environmental Sciences

PAKISTAN ACADEMY OF SCIENCES
ISLAMABAD, PAKISTAN

Proceedings of the Pakistan Academy of Sciences: Part B Life and Environmental Sciences

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Annual Subscription: **Pakistan:** Institutions, Rupees 8000/- ; Individuals, Rupees 4000/- (Delivery Charges: Rupees 300/-)
Other Countries: US$ 200.00 (includes air-lifted overseas delivery)

HEC Recognized; Scopus Indexed

Published by **Pakistan Academy of Sciences**, 3 Constitution Avenue, G-5/2, Islamabad, Pakistan
Email: editor@paspk.org; **Tel:** 92-51-9207140; 92-51-920 6770; **Websites:** www.paspk.org/proceedings/; www.ppaspk.org

Printed at **Graphics Point.,** Office 3-A, Wasal Plaza, Fazal-e-Haq Road, Blue Area, Islamabad
Ph: 051-2806257; E-mail: graphicspoint16@gmail.com

PROCEEDINGS OF THE
PAKISTAN ACADEMY OF SCIENCES: PART B
Life and Environmental Sciences

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 117-132 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)947

Pakistan Academy of Sciences

Review Article

An Insight into Male Infertility: A Narrative Review

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Abstract: Male infertility is a widespread health issue globally, frequently remaining untreated due to stigmatization and the challenges in diagnosis and treatment. This review presents a comprehensive update on the literature covering key aspects such as causes, diagnostic techniques, and treatment options for managing male factor infertility. It includes an in-depth analysis of global infertility data, using resources from the World Health Organization, Web of Science, Google Scholar, Elsevier, Medline, PubMed, and Scopus databases to gather relevant articles on male infertility. A total of 41 articles from 2000-2023 were reviewed. High throughput techniques, along with sophisticated assays, are being employed for accurate diagnosis. Surgical procedures such as testicular sperm extraction, vasovasostomy, vasoepididymostomy, sperm retrieval techniques, and non-surgical procedures including sclerotherapy, gonadotropin-releasing hormone therapy, and antiestrogens are available to treat infertile males. Additionally, in recent years, flavonoids have been extensively explored for their antioxidant, anti-inflammatory, immune-stimulating, anti-apoptotic, anticarcinogenic, anti-allergic, and antiviral activities. These properties of flavonoids are being investigated for their potential to address biological mechanisms underlying anomalies such as spermatogenesis disturbance and sperm quality decline. This review serves as a comprehensive guide to better understand the etiologies and treatment modalities of male factor infertility, ultimately facilitating affected individuals in making informed reproductive choices.

Keywords: Male Infertility, Genetics, Environmental Factors, Diagnosis, Treatment, Flavonoids.

1. INTRODUCTION

Male infertility is defined as the inability to produce a pregnancy despite regular intercourse without contraception for at least one year. This is least considerate disorder in many societies. Regardless of how clinical experts might characterize infertility, couples mostly don't characterize themselves as 'infertile' or present themselves for treatment unless they embrace parenthood as a desired social role [1]. Globally, male infertility is under-reported particularly in male dominant societies. Male infertility is a common problem that affects approximately 48.5 million couples worldwide and was predicted to be largely genetic in origin [2]. However, other studies reported involvement of epigenetics in disease manifestation including DNA methylation, histone tail modifications, and non-coding RNAs [3]. In addition to genetics and epigenetics factors, environmental and lifestyle elements significantly contribute to male infertility. Exposure to pollutants, occupational hazards, smoking, alcohol consumption, substance abuse, poor dietary habits, obesity, stress, infections and certain medications can adversely affect reproductive health.

Sperm production is a highly complex process and genetic variant/s may compromise normal sperm development and maturation. Various studies to investigate genetic basis in both humans and mice revealed the involvement of various crucial pathways for male infertility, including sexual differentiation, development of the genitourinary system and gametogenesis. According to Jackson Laboratory's Mouse Genome Informatics database

Received: July 2023; Revised: April 2024; Accepted: June 2024
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more than 600 male infertility genes and 2300 testis-enriched in human have been reported. In addition to genetics and epigenetics, the causes of male infertility are numerous. Blockages in the reproductive tract and hormonal imbalances can cause infertility [4-7].

Despite the complex nature of male infertility, it can be treated in some cases through various surgical procedures such as testicular sperm extraction, vasovasostomy, vasoepididymostomy, and sperm retrieval techniques. Non-surgical methods like sclerotherapy and gonadotropin-releasing hormone therapy are also utilized. Currently, the global search for an inhibitor of male reproductive system dysfunction continues. Among many natural products, flavonoids have been extensively investigated as potential inhibitors for treating male infertility [8]. Flavonoids are the most prevalent and well-studied polyphenols in human diet. There are approximately 4000 flavonoid varieties reported so far commonly found in seeds, citrus fruit, olive oil, tea, and red wine [9]. Flavonoids are a broad group of natural antioxidant compounds with flavan nucleus and a benzo-γ-pyrone structure having a basic C6-C3-C6 phenyl-benzopyran backbone. Interestingly, these amphipathic molecules can penetrate the lipid bilayer of membranes, thus providing possible protection for the entire spermatozoa and acrosome membrane [10]. Hence, flavonoids can prevent oxidative damage and facilitating the acrosome reaction of spermatozoa necessary for fertilization.

This review discusses the causes of male infertility and outlines the diagnostic and treatment strategies available. Additionally, it explores the therapeutic effects of flavonoids on male reproductive system dysfunction.

2. METHODOLOGY

A literature survey was conducted for meta-analyses and systematic reviews related to male infertility, using Google Scholar (https://scholar.google.com/), MEDLINE (https://medlineplus.gov/), and PubMed (https://pubmed.ncbi.nlm.nih.gov/) to search for articles published in English in peer-reviewed journals. Keywords used in the search included "male infertility," "genetic causes," "environmental causes," "diagnosis," and "treatment".

Inclusion criteria: Initially, a total of 59 articles related to male infertility from 2000-2023 were selected. The titles and abstracts of these articles were screened to determine which studies met the inclusion criteria. The full texts of the shortlisted articles were then reviewed, and data were extracted and pooled. Ultimately, 41 articles were chosen based on their relevance to the topic or the availability of the full text.

Exclusion criteria: Studies containing ambiguous or duplicated data from the same author across different journal articles were also excluded. Additionally, articles that were not available in full-text format and were inaccessible through Sci-Hub (https://sci-hub.se/) were excluded from the review.

3. ETIOLOGY

3.1. Genetic

Over 2000 genes have been linked with spermatogenesis and spermiogenesis, any mutation in these genes can result in abnormal production of sperms. All these mutations have been categorized as quantitative (that affect the number of sperm) and qualitative (which causes abnormalities within the sperm) spermatogenesis disorders (Figure 1). Research has indicated that there is a 15% prevalence of genetic abnormalities in males experiencing azoospermia (no sperm ejaculation) and severe oligozoospermia (low sperm count in semen). The primary genetic contributors include microdeletion on the Y chromosome, abnormalities in karyotype and mutation sin the cystic fibrosis transmembrane conductance regulator (CFTR) [11]. Apart from various Y chromosome microdeletions, abnormalities in X-chromosome can also lead to male infertility [12].

The Y chromosome was partially sequenced about two decades ago which includes 53.8% sequencing of the GRCh38 Y chromosome. The Y chromosome comprises of euchromatin and heterochromatin regions. The Euchromatin region (around 25 Mb) comprises of two pseudoautosomal regions, PAR1 and PAR2, that actively recombine with the X chromosome. The remaining chromatin (around 22 Mb) is categorized into X-degenerate regions (8.6 Mb), X-transposed regions (3.4 Mb), and ampliconic regions (9.9 Mb) with distinct

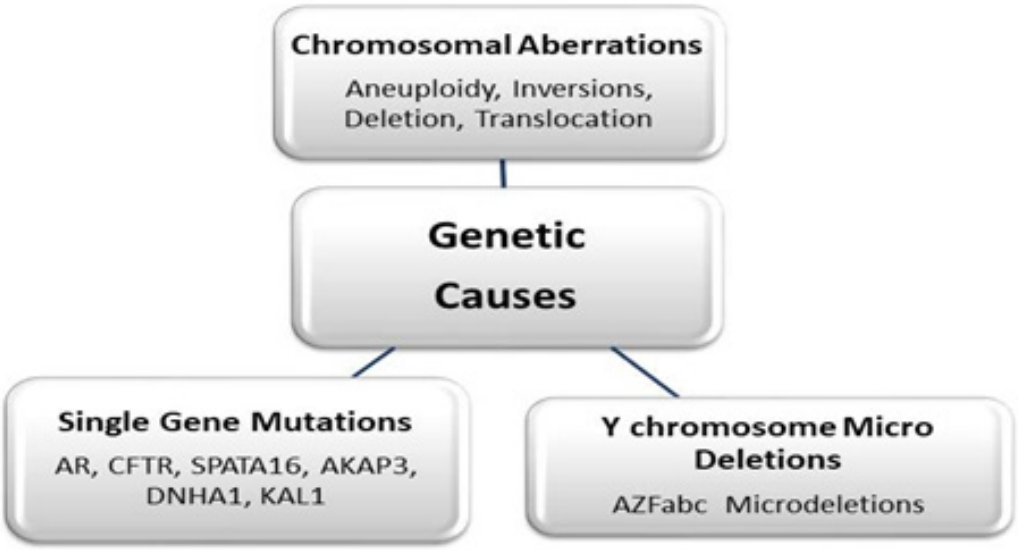

Fig. 1. Summary of various genetic causes in male infertility.

evolutionary origins [13] as shown in Figure 2. The last three segments: X-degenerate regions, X-transposed and ampliconic, are collectively known as the male specific regions of the Y-chromosome (MSY). Since these regions lack homologous sequence, they have a single copy that is directly inherited from the father[14]. These regions have highly replicative genes due to the presence of long palindromic duplicates (high identity of 99.9%) that are involved in spermatogenesis [15]. Due to these repetitive sequences this region is highly prone to intra and inter chromatid-reciprocal recombination which can lead to severe phenotypes such azoospermia and oligozoospermia. The second most common genetic aberration that can occur and leads to male infertility is the microdeletion of Yq (long arm of Y chromosome). It is observed in 1% to 7% of males experiencing severe oligozoospermia and in 13% males with azoospermia [16, 17]. Similarly,

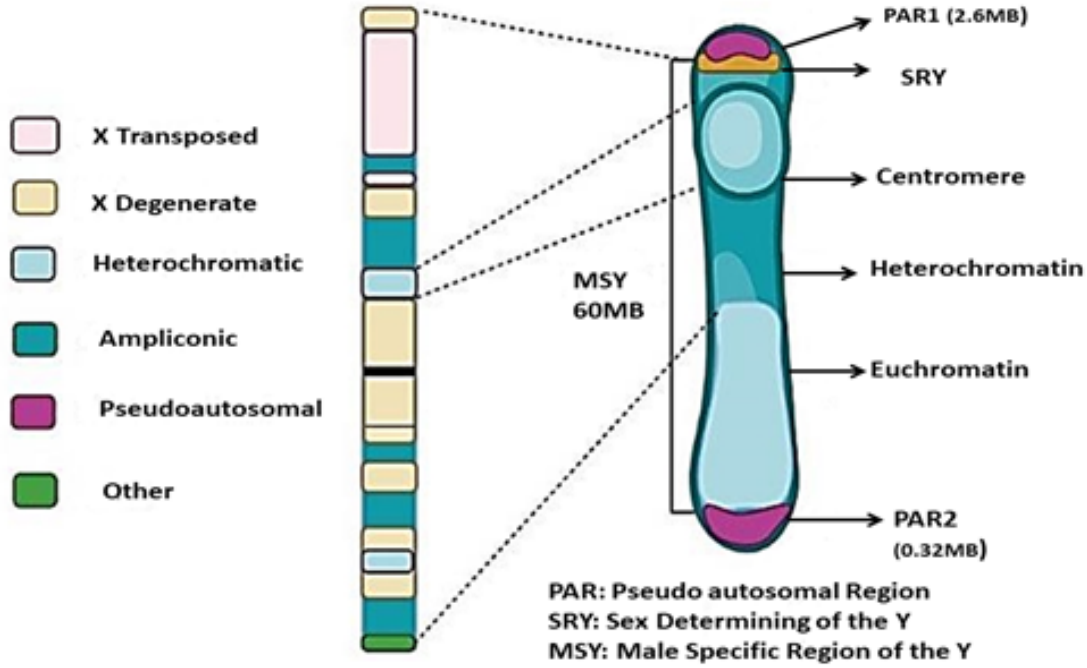

Fig. 2. Structure of Y chromosome.

the Y-chromosome has an azoospermia factor region on its long arm which has three domains: AZFa, AZFb and AZFc. Studies have revealed that complete deletion of AZFa region leads to loss of DEAD box protein 3, Y chromosomal and ubiquitin specific peptidase 9 Y-linked genes due to which there is total loss of Sertoli cells only hence absence of sperms. If the AZFb region is completely deleted affects the Y-chromosome RNA recognition motif 1 (RBMY1 and PTPN13 like Y-linked (PRY) clusters, due to which the spermatocytes are stuck in mitotic arrest during its primary stage and never fully develops. AZFc mutations are more heterogeneous as compared to AZFa and AZFb region deletion [18].

In addition to Y chromosome deletions, mutations in the X chromosome have also been reported to cause infertility in males. The primary function of sex genes is in sexual differentiation [19]. The X chromosome is heterozygous expressed in men but it does encode for multiple testicular proteins and also has been found to play a role in spermatogenesis [20]. Mutation sin the X-linked testis expressed 11 (TEX11) gene have been linked to male infertility, particularly azoospermia [21, 22]. Similarly, mutations in the human reproductive homeobox (RHOX) gene cluster have been associated with male infertility, with aberrant RHOX promoter methylation strongly linked to abnormal sperm parameters [23, 24]. Protein encoded by TEX11 plays a significant role in pathogenesis of azoospermia while RHOX gene is expressed in germ cells. Mutation in RHOX such as RHOXF1 and RHOXF2/2B causes severe oligozoospermia [21, 25]. Structural defects leading to male infertility are rare, accounting for only 1-2% of all cases [26]. These defects are most commonly caused by a birth injury to the penis or testes. Trauma to the testes can also occur as a result of accidents or sports injuries [27]. Deletion or addition of a whole chromosome during meiosis can lead to abnormal number of chromosomes due to paternal or maternal nondisjunction. The most common structural aberrations found in men suffering from infertility are reciprocal, inversions, and Robertsonian translocation. Oligozoospermic men, who have a reduced sperm count, are more likely to have autosomal structural rearrangements, with a 10% chance of such abnormalities [28]. These rearrangements can lead to defects in spermatogenesis and abnormal segregation at meiosis, contributing to the reproductive phenotype of these men [29].

However, certain karyotype abnormalities (variations in the number or structure of chromosomes) are not detrimental but are silent mutations hence resulting in normozoospermic (normal sperm) men, the prevalence for such cases are mere 0.75 to 1.0%. Therefore, the number for deleterious karyotype mutations is approximately high i.e., 15% for nonobstructive azoospermia and 4% for severe oligozoospermia [30].

Some very obvious indicators for a karyotype abnormality are low sperm count (less than 5 million per mL), recurrent miscarriages, fetal malformation or cognitive developmental disability. Chromosomal aberrations are not only numerical but structural too and they not only necessarily result on Y chromosome but can also involve X chromosome and autosomes as well. Klinefelter syndrome (KS; 47, XXY) is a chromosomal abnormality that results in aneuploidy, leading to nonobstructive azoospermia in men [31]. The prevalence for this disorder is 14.8% men with azoospermia, 5.4% men with severe oligozoospermia, and 4% of all overall male infertility [32] Whilst, there are other variants present too, about 20% of affected men have higher levels of structural defect due to 47XXY/46, XY [33]. Studies have reported that sperm obtained through microsurgical testicular sperm extraction method (micro-TESE) offers higher sperm count as compared to conventional testicular sperm extraction (TESE), especially with men that have nonobstructive azoospermia this also includes Klinefelter syndrome patients [34, 35]. In non-mosaic Klinefelter patients, the sperm aneuploidy rate is 2-25% while in mosaic Klinefelter patients it is 1.5%-7%. This increases the chances of affected individuals to have offspring [36].

Another condition, 46, XX Male Syndrome also known as de la Chapelle syndrome, is a rare genetic condition in which an individual has two X chromosomes (typically found in females) but exhibits male characteristics. This occurs due to the presence of male-determining genes, usually transferred from the Y chromosome to one of the X chromosomes through a process called translocation. At the time of birth such patients have normal external male genitalia; the condition is only

noticeable upon puberty because of infertility and hypogonadism. The testes of these patients contain hyalinized seminiferous tubules lined with Sertoli cells only (SCO). It has been observed that about 90% of the affected individuals have an SRY gene translocated on the terminal end of the short arm of X chromosome or a rare autosomal chromosome [37]. Generally, no treatment is available for such genetic disorders except symptomatic management. Fortunately, genetic screening and genetic counseling could be an opted as a possible solution. Along with this, patients with aneuploidy opting for in vitro fertilization should also consider preimplantation genetic testing to ensure a healthy offspring.

3.2. Hormonal Imbalance

Hormones play a key role in the reproductive process, and imbalances can lead to fertility problems. Hormonal defects account for about 15% of all cases of male infertility [38] . They are most commonly caused by a problem with the hypothalamus or the testes, but they may also be due to a problem with other endocrine male infertility. It is important to note that hormonal problems usually affect sperm quality, not the total number of sperm [39]. A hormonal imbalance caused by too much prolactin may lead to low sperm production as well as undescended testes or other problems with sexual development [40]. Too little testosterone or an abnormal ratio of testosterone to estrogen may result in fatigue, loss of sex drive, erectile dysfunction, and reduced sperm production [41]. There are many different types of hormone imbalances that can cause infertility, including: Hypothyroidism – an underactive thyroid gland [42]. The symptoms include fatigue, weight gain, depression, and slowed heart rate and muscle weakness. Patients with hypothyroidism often have abnormal levels of prolactin in their blood [43]. Hyperprolactinemia – abnormally high levels of prolactin in the blood due to a problem with the hypothalamus or pituitary gland that causes low testosterone production. Low testosterone can lead to infertility [44]. Hyperthyroidism – an overactive thyroid gland that causes the body to produce too much of the hormone thyroxine. Patients with Diabetes have a higher risk of developing fertility problems due to changes in hormones and sperm quality. There is also evidence that diabetes may damage testicles and reduce testosterone production, thus causing infertility [45]. Hormonal medications can interfere with sperm production and lead to the development of female sex characteristics such as breast enlargement. Exposure to certain chemicals or radiation may cause a hormonal imbalance in some men that leads to fertility problems [46]. According to cancer research, exposure to high levels of heat or certain chemicals may cause a man's testicles to shrink. Shrunken testicles are unable to produce sperm, which leads to infertility. Heavy exposure to chemicals such as lead or cadmium can damage sperm and make them less capable of fertilizing the egg [47]. Therefore, the high mutation rates resulting in extensive structural polymorphism among human Y chromosomes and considering the multifactorial nature of male infertility, developing new diagnostic panels is essential for transforming the current landscape of prevention, diagnosis, and management [48, 49].

3.3. Epigenetics

The term epigenetics can be defined as changes in phenotype caused by methods and mechanisms other than changes in DNA sequences, these can be DNA modifications, histone modifications, and RNA interference [50].

3.3.1. DNA methylation in male infertility

DNA methylation predominantly occurs in CpG islands and is controlled by DNA methyltransferases (DNMT) and can influence gene expression by suppressing transcription [51]. Correct DNA methylation is essential for various cellular processes crucial for male fertility, including spermatogenesis and chromatin stability [51, 52]. Recent evidence depicts the connection between proper DNA methylation and male fertility. Abnormal DNA methylation patterns are observed in spermatozoa of infertile men, impact sperm quality, motility, and DNA integrity [53–55]. Hypermethylation of promoters of specific genes such as MTHFR, IGF2, H19, PLAG1 and SNRPN, is associated with poor sperm quality and an increased risk of infertility [56]. Spermatogenesis a critical process for male fertility is influenced by DNA methylation abnormalities in genes H19, MEST, and RHOX clusters, leading to conditions such oligozoospermia [57, 58]. Additionally,

hypomethylation of H19 has been observed in men with teratozoospermia [59, 60].

3.3.2. Histone modification in male infertility

The intricate landscape of nucleosome, the fundamental units of chromatin, is a key player in male fertility. Consisting of DNA wrapped around histone octamer, including histones, H2A, H2B, H3 and H4, this structural organization lends rigidity to chromatin. Crucial to this dynamic is the realm of histone modifications, a cascade of covalent post-translational changes primarily occurring on the lysine-rick tail of histone proteins, notably H3 and H4 [61]. Histone acetylation is a reversible process catalyzed by acetyltransferases and deacetylases, acting as a significant regulator. This modification neutralizes positive charge of the amino acid on histone tail therefore reducing DNA affinity and facilitating an open chromatin structure for transcription. It has been found that H4 hyperacetylation (Hypac-H4) is a crucial modification during spermatogenesis, observed spermatogonia, spermatocytes and spermatids. Histone modifications such as H4K8ac, H4K5ac, H4K20me2, and H4me exhibit distinct patterns during various stages of spermatogenesis. Studies have been conducted that elaborate the intricate relationship between Hypac-H4 and impaired spermatogenesis [62]. Histone methylation, another facet of epigenetic regulation, dictates the activation or repression of chromatin states. Diverse patterns of histone methylation have been unveiled in human spermatozoa, and abnormal methylation is associated with severe damage to the spermatogenesis [63]. Additionally, histone phosphorylation has also been found to be involved in transcriptional activation and chromatin rearrangement during spermatogenesis. Defects in histone phosphorylation are involved in sperm dysfunction and male infertility issues. It has also been found that histone phosphorylation can regulate several biological events including mitotic/meiotic chromosome condensation, activation and inactivation of genes transcription, chromatin remodeling and double-strand DNA break repairs (DSB) which are all essential processes in sperm development [64]. In addition to this the process of histone-to protamine exchange during spermatogenesis marks as a crucial juncture. The somatic histones are replaced by protamine, the core of the spermatids condense and the process ensure the safety of sperm genome against the rigors of fertilization [65]. Recent studies have indicated that epigenetic modifications of histone play a pivotal role in orchestrating histone-to-protamine exchange in human spermatozoa [64].

3.3.3. Chromatin remodeling in male infertility

Chromatin remodeling is a dynamic process which consists of protein complexes like SWI/SNF, ISW1 and MI2 which alter the nucleosome location and structure through ATP-dependent process [66]. This process has been found to be crucial in condensing chromatin in spermatozoa to transmit epigenetic information to the embryo [67]. Previous studies revealed that correct DNA packaging during spermatogenesis, where 85% histones are replaced by protamine's is vital [68, 69]. Aberration in this process, such as mutation in Calcium/calmodulin-dependent protein kinase 4 (CaMK4), which participates in phosphorylation of protamine 2 has been linked with impaired spermatogenesis and male infertility [70]. In addition to this, PRM1 md PRM2 which are key proteins in sperm function and fertilization are crucial and their haploinsufficiency can lead to reduced protein levels that can result in abnormal chromatin structure and a damaged DNA in sperm [58]. The PRM1 and PRM2 ratio needs to be tightly regulate, any deviation has the potentially to impact the sperm quality, DNA integrity and male fertility [71]. Studies have also associated abnormal histone H4 acetylation to impaired spermatogenesis, since H4 hyperacetylation is crucial for histone to protamine transition [60, 72]. It has been observed especially in conditions like Sertoli cell-only syndrome (SCOS) [73].

3.3.4. Genetic imprinting in male infertility

Genetic imprinting is an epigenetic process which dictates allele expression in a parent-of-origin-specific manner through CpG island methylation changes. It has been observed that men suffering from male infertility have aberrations in imprinted genes like GTL2 and H19 indicating the significance of genetic imprinting during spermatogenesis [58, 74, 75]. It has been observed that fertile males have high IGF2/H19 imprinting control region 1 (ICR1) methylation levels while reduced MEST methylation is linked to low sperm counts, this

serves as a strong indicator of sperm quality in infertile males [76]. Assisted reproductive techniques (ART), including ICS1, IVF and ROSI are linked with an increase prevalence of imprinting defects, potentially impacting embryonic development and elevating the risk of infertility and congenital abnormalities in offspring [77]. Optimizing ART techniques and ensuring long-term follow-up for ART offspring are crucial for comprehensive clinical understanding [78].

3.3.5. miRNAs in male infertility

Epigenetic modifications including non-coding RNAs like microRNAs (miRNA), play a crucial role in male infertility. miRNA make a substantial portion of cellular RNAs and regulate post-transcriptional gene expression. Dysregulation of miRNA expression in sperm cells has been associated with severe abnormalities in these cells and can impact subsequent generation [79]. Experimental studies have highlighted transgenerational inheritance of altered DNA methylation and non-coding RNA in sperm, emphasizing the importance of understanding miRNA involvement in male infertility [80]. Eating a healthy diet, exercising regularly, maintaining a healthy weight, and avoiding toxins and excessive alcohol intake are all good steps toward improving fertility. Reducing stress levels is also important, and may be accomplished through exercise, relaxation techniques, or simply spending time with friends and family. Medications such as antidepressants and high blood pressure medicine can negatively affect fertility by reducing sex drive or causing erectile dysfunction that makes intercourse difficult if not impossible. Men who smoke are more likely to have low sperm count than non-smokers [81]. Obesity also increases the risk for diabetes which can cause testicular damage that impairs sperm production or causes sexual dysfunction that makes intercourse impossible [82]. A healthy diet with plenty of fruits, vegetables, lean meats, whole grains, and other unprocessed foods will go a long way toward preventing obesity [83]. Excessive alcohol intake has been associated with reduced sperm counts as well as reduced fertility in general by increasing estrogen levels in men which may lead to fertility problems [84]. Environmental toxins such as heavy metals and pesticides can also cause epigenetic changes that lead to male infertility [85]. Treatments for male infertility depend on the underlying cause and may include lifestyle changes such as quitting smoking, losing weight, and eating a healthy diet to reduce obesity-related testosterone reduction and improve sperm count; supplements such as vitamins or antioxidants to treat hormone imbalances or vitamin B12 shots to increase sperm production [86]. For men with more serious fertility problems, assisted reproductive technologies such as in-vitro fertilization (IVF) may be necessary [87].

4. DIAGNOSTIC TECHNIQUES

Next-generation sequencing, biomarkers, enzymatic tools, and miRNA technology are widely used for diagnosis of male infertility [88]. Moreover, epigenetic studies support an in-depth understanding of this disease other than the genetic contribution; this allows the researchers to diagnose male infertility at an early stage [89]. There are multiple conventional diagnostic approaches for male infertility that help the physician to select appropriate treatment methods for patient [90]. The scrotal ultrasound test is used in which high-frequency sound waves produce images of the body. This test provides information regarding the varicocele-related problems in testes [91]. Transrectal ultrasound provides the sample for the prostate, hormone testing is performed to diagnose the abnormalities in organ systems that contribute to male infertility. Post-ejaculation analysis is performed to determine the sperm in urine and indicates that the sperm travels back into the bladder rather than into the penis during ejaculation [92]. A blood test (Y-chromosome microdeletion test) is performed to reveal the subtle changes in the Y chromosome. This test shows sign of genetic abnormality. Testicular Biopsy involves the sample removal from the testicle with the use of a needle. These test results are particular helpful in cases of obstructive azoospermia, since they help to identify whether the absence of sperm in the ejaculate is due to a blockage in the reproductive tract. A positive report of the biopsy means that presence of sperms has been identified in testicular tissue, which indicates that the absence of sperm in ejaculate is likely due a blockage in the reproductive tract. [93–96]. Specialized sperm function tests are the accumulation of the series of tests that investigate the survival of sperm after ejaculation, also how well sperm penetrates an egg.

4.1. Next-Generation Sequencing

Genetic investigation has made exceptional advances owing to the availability of NGS platforms. Contrary to single gene mutation identification through exon-by-exon amplification and Sanger sequencing, NGS enables the interrogation of large panels of genes in a single experiment and at a reasonable cost. Also, the cost of whole exome sequencing and whole genome sequencing has dropped in the last decades, therefore, novel genes causing male infertility can be rapidly identified [97].

4.2. Sperm Analysis

Sperm analysis is done by different methods. In which sperm morphology, motility, and size of sperm are analyzed. Microfluidic techniques were combined with high-speed imaging that was given the full 3F swimming patterns of the sperm that was present in the bulk fluid mixture. This technique shows an in-depth study of the chemo-taxis and rheotaxis of the sperm (34). Genetic imbalances are determined by using fluorescence in situ hybridization (FISH) which identifies the abnormal number of chromosomes that are analyzed for this disease. FISH uses fluorescent tag to particular DNA elements to determine the aneuploidies which are because of the mitotic errors [39, 98–100]. While FISH is primarily used to identify chromosomal abnormalities, it can also be employed to estimate the number of sperm with specific genetic traits or abnormalities Other emerging techniques are super-resolution microscopy, digital holography, and next-generation sequencing [101]. Computer-aided sperm analysis (CASA) can be utilized to measure sperm motility. Sperm RNA-based Analysis was done in which different types of RNAs including non-coding RNA (siRNA, piRNA, miRNA, IncRNA, and Tisense RNA), and coding RNA. It was seen that these RNA affects the fertility of the men, and the RNA amount in the somatic cells was much higher than in spermatozoa, therefore somatic cells were considered to be removed from the transcripts of sperms. RNA sequencing and Microarray profiling were used to identify the transcription levels of spermatozoa [102].

4.3. Assays

Genetic and epigenetic markers are used to diagnose male infertility. Presently available tests include the DNA fragmentation index, anti-sperm antibody assays, sperm fluorescence in situ hybridization, and other sperm functional tests. The basic challenge in finding the predictive biomarkers is the poor diagnostic capability of all existing assays. Novel male infertility biomarkers are based on emerging research areas such as proteomics, epigenetics, genomics, metabolomics, and transcriptomics. As one of the leading biomarkers for male infertility Reactive Oxygen Species (ROS) has gained interest, because of its wide application in diagnosis of male infertility. For the metabolomics approach, ROS in semen is most widely used [103]. An anti-sperm antibody (ASA) assay is used for evaluating male infertility. It confirms the presence of the immunoglobulins that bind with the patient's sperm. These immunoglobulins clumped with the male sperm and reduced the motility and function of the sperm. This assay is reported to not provide efficient, and reliable results. Another assay that is used is the DNA fragmentation index (DFI) assay; this assay measures the integrity of the sperm DNA. As exposure to certain kinds of stress issues has a bad impact on fertilization, and embryo development [104]. Other assays that were used in different studies were protein-based assays ECM1, ACCRV1, and TEX101. All of these assays are reported to be useful in the diagnosis of sperm concentrations, and different aspects of sperm survival such as motility, morphology, size, and others. But there is a need to develop an efficient assay, and that does not require stepwise testing [105]. Therefore, these diagnostic methods can be utilized to decide the treatment options for affected individuals.

5. TREATMENT STRATAGIES

There are several treatments methods available to treat male infertility which increase sperm production and improves fertility rate.

5.1. Surgical Treatments

In cases of infertility that can be attributed to epigenetic factors, surgical procedures such as testicular sperm extraction (TESE) may be used to recover sperm from unreceptive areas. However, this approach is currently only feasible for a few types of cancer patients and is not readily available [106].

Sperms retrieval for Males who have Obstructive Azoospermia and varicocele can be treated by using surgical treatments. Microdissection testicular sperm extraction method is also a surgical method and after this surgery 70-90% of men returns to the normal ejaculation of sperms [107].

5.1.1. Vasovasostomy

Vasovasostomy (VV) is a surgery that involves the reconnection of the vas deferens to restore sperm flow. This surgery is often used as part of infertility treatment procedures for men with obstructive azoospermia, an otherwise irreversible condition caused by occlusion of the vasa deferentia. Vasovasostomy can be planned based on the patient's age, comprehensive medical history, family medical history, overall health status, and psychological condition. Vasovasostomy process is done under mild anesthesia, the part under examination is thoroughly shaved then the patient is laid down in comfortable position both vasa are carefully examined in most of normal cases only one side is needed to be repaired [108, 109].

5.1.2. Vasoepididymostomy

Vasoepididymostomy (VE) is a sperm retrieval technique used to bypass an obstruction in the vas deferens and epididymis, restoring fertility to men who suffer from non-obstructive azoospermia [97]. The procedure involves the reattachment of the epididymal tubule and spermatic cord to create a longer path for sperm flow, thus increasing chances of successful fertilization. Vasoepididymostomy is a complex surgery as compared to VV; it is only performed if there are no sperms that are visualized after the analysis of fluid taken from vas deferens. The selection of procedure depends upon the quality of sperms [110].

5.1.3. Sperm retrieval techniques in OA (Obstructive Azoospermia)

Sperm retrieval techniques are another option for men with non-obstructive azoospermia who wish to conceive children through IVF. The sperm retrieval method is commonly used for patients who do not undergo reconstruction; the main aim of this technique is hankering of sperms from testis and epididymis percutaneous or opens [111]. Percutaneous Epididymal Sperm Aspiration (PESA) is a straightforward procedure that does not require an operating room, anesthesia, or specialized microsurgical staff. In this process, a small needle is used to cannulate the epididymis and extract some epididymal fluid, which is then analyzed for sperm. If necessary, the procedure is followed by Testicular Sperm Extraction (TESE) [112]. The TESE procedure may also be employed to locate and extract sperm from the testes of patients with obstructive azoospermia. Sperm from these procedures can be used for ICSI to fertilize eggs from female partners. In TESE after making a small incision in the tubules for the extraction of sperms. TESE success rate in men with NOA is 20-45% [106]. Microsurgical testicular sperm extraction (MicroTESE) is another technique in which a microscope is used and more keen extraction is done under microscopic conditions in sperm retrieval the success rate of MicroTESE is up to 60% [113]. MESA Microsurgical epididymal sperm aspiration is usually performed under local anesthesia, where the sperm is aspirated from the different ducts or epididymis under an operating microscope [114].

5.2. Nonsurgical Treatments

5.2.1. Sclerotherapy

Sclerotherapy means sclera (Hardness) and therapy (treatment) Sclerotherapy is a non-surgical treatment in which blockage in the vessels is addressed. It is an effective treatment for obstructive azoospermia where spermatic cord occlusion is caused by blood vessel malfunction. The procedure involves injecting a substance known as a sclerosant in case of NOA the injection of ethanol into these blood vessels to dissolve them. This method can restore fertility in patients with unilateral or bilateral non-obstructive azoospermia, allowing them to conceive children through IVF [115].

5.2.2. Gonadotropin-releasing hormone therapy

Gonadotropin-releasing hormone (GnRH) is a peptide hormone that regulates follicle-stimulating hormone and luteinizing hormone. Excessive levels of these hormones can cause the degeneration and death of germ cells, ultimately leading to infertility in men and women. GnRH agonists

such as leuprolide acetate can be administered to suppress the release of these hormones, increasing the survival rate of sperm in men with azoospermia. GnRH agonist therapy is also used as a treatment for chronic testicular pain [116].

5.2.3. Antiestrogens

Selective estrogen receptor modulators (SERMs) such as tamoxifen and clomiphene can be used in conjunction with gonadotropin and antiandrogen treatment to stimulate spermatogenesis in men with non-obstructive azoospermia [117].

5.2.4. Aromatase inhibitors

Aromatase inhibitors are being developed as a treatment for male-factor infertility, acting to eliminate the levels of estrogen within the body. Furthermore, inhibiting the activity of aromatase can block the conversion of testosterone into estrogen, thus increasing the amount of free testosterone in men [118].

5.2.5. Flavonoids

Flavonoids are low molecular weight polyphenols ubiquitously synthesized by green plants that may show various pharmacological attributes according to their chemical structures. So far, different flavonoids like quercetin (Q), rutin (R), naringenin (N) and epicatechin (E) have shown their potential in improving sperm motility and viability. Specifically, rutin has been reported to improve the kinematic parameters of post-thawing sperm, as well as its fertilizing characteristics. Quercetin, which is a reddish pigment found in 70 plant species and is present in many plant-based food products [119]. Flavonoids can also be found abundantly in apple skins, red wine, and red onions. Quercetin has been mostly explored for its free radical scavenging and metal chelating properties [120–122]. Furthermore, it can be easily taken from apple skins, in red wine and in red onions. Q has been mostly explored for its free radical scavenging and metal chelating properties [120, 121]. There are multiple factors like Oxidative stress, apoptosis, inflammation which may prompt male reproductive system dysfunction. Studies suggest that oxidative stress is observed in around half of all infertile men. These factors may lead to small testis in the weight and may disrupt testicular structural which can eventually lead to inhibition of spermatogenesis with compromised sperm quantity and quality [9, 10]. Similarly, quercetin lowered the endocrine and testicular abnormalities caused by the heavy metal cadmium in male rats. Apigenin, EGCG, and luteolin have been shown to elevate gene expressions of steroidogenic acute regulatory protein (StAR), cytochrome P450 11A (CYP11A), and CYP17A. These effects are beneficial for restoring Leydig cell function and testosterone secretion [123, 124]. Various in vitro and animal studies have reported therapeutic potential of rutin in male infertility but the exact mechanisms are yet to be explored. Contrary to a lot of therapeutic potentials of flavonoids, some studies have claimed little antioxidant activity due to limited bioavailability of rutin in in vivo conditions [125]. Moreover, male Infertility can be caused due to metal toxicity which eventually results in ROS induction [126]. Consequently, antioxidant therapy is an encouraging strategy for treatment of individuals with heavy metal poisoning [127]. Despite carotenoids and vitamin E, flavonoids provide protection against metal toxicity [128]. It has been suggested that three flavonoids, rutin, naringin, and kaempferol have been shown to restore motility of AlCl3 -, CdCl2 -, and PbCl4 exposed sperm cells. Whereas, other two flavonoids, catechin and quercetin, had no positive effects on motility of metal-exposed sperm; rather, they decreased sperm motility compared to untreated control samples [129]. Therefore, to translate such findings into clinical reality, more studies should be conducted to see how these flavonoids could be utilized on larger scale to deal with sperm abnormalities.

6. CONCLUSIONS

Male infertility is a very complex biological condition that can be caused by different factors. Despite, involvement of various factors; genetic, environmental, and psychological there exist many treatment options for patients, and most cases can be treated successfully. Treatment options include medication, dietary supplements like rutin, surgery, and assisted reproductive technologies (ART). In some cases, lifestyle changes such as quitting smoking or drinking may also be recommended. Society should also encourage men to get checked for infertility.

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 133-148 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)826

Pakistan Academy of Sciences

Review Article

Yield Decline and Resistance Development in Sucking Pests of Cotton in the Context of Unwise Spraying Techniques

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Abstract: Over 100 countries are producing cotton, which provides raw materials to the industry and employment opportunities for the people. Limiting the cost of production, conserving the ecosystem, and improving the cotton yield are key ingredients of sustainable cotton production. The inception of transgenic cotton (Bt varieties) improved yields, curtailed pesticide uses and promoted environmental safety. Over the years, Bt varieties of cotton have lost resistance against bollworms, and yield is declining. Unwise application of pesticides is associated with the development of resistance to sucking pests of cotton. The present review was conducted to undertake (i) the background of cotton production in the world, (ii) varietal development in cotton, (iii) problems (increasing susceptibility) in Bt varieties (iv) reliance on pesticides, and (v) myths of pesticides applications followed by pesticides knowledge among farmers. The cotton varieties which are resistant against sucking pests should be introduced. In addition, the promotion of biopesticides and fostering the adoption of Integrated Pest Management Approaches could be effective for the management of sucking pests. Farmers must be trained in site-specific and accurate spraying techniques by agricultural extension and plant protection departments. Accurate spraying techniques will not only improve pest control but also help in curtailing the environmental pollution being caused by the excessive use of pesticides in cotton.

Keywords: Sucking Pests, Resistance, Susceptibility, Bollworms, Ecosystem, Biopesticides.

1. INTRODUCTION

The world is producing around 25 M tons of cotton. India, China, the United States, Pakistan, Brazil, Australia, Uzbekistan, Turkey, Turkmenistan and Burkina Faso are the top ten cotton-producing nations in the world [1]. Globally, the total cotton production was expected to reach 121.6 million bales in 2021-22 [2]. *Gossypium hirsutum*, *Gossypium babadense*, *Gossypium arboretum*, and *Gossypium herbaceum* are four commonly grown types of cotton. A major proportion of the cotton area is devoted to Bt cotton [3]. The global cotton production is forecasted to grow by 1.6% and reach 30.6 million tons by 2031. The average yield of cotton is also to grow at 1.3%, which is likely to back the increase in global production of cotton [4].

Cotton production is much needed for the world for many reasons, including an addition to the economy and support to livelihoods [5]. Conventional cotton was not very effective due to severe susceptibility to pests. Transgenic cotton helped to combat the pest's attack. Since the inception of transgenic cotton, pesticide use patterns have changed, production increased, and the natural enemy population jumped high, followed by improved land use efficiency and productivity [6]. However, transgenic cotton has started showing susceptibility towards pests; moreover, pests have developed resistance. This transition is not only decreasing production but also increasing the cost of pesticide application along with the occurrence of environmental degradation [7]. The improper application of pesticides seems more critical in developing resistance and leading transgenic cotton to failure.

With a population of over 212.82 million and a population growth rate of 2.4%, Pakistan is the 6th most populous nation. Agriculture contributes to 18.5% of the country's GDP and provides

Received: March 2023; Revised: April 2024; Accepted: June 2024
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livelihood support to 38.5% of the population, making it a crucial sector for the country [8]. Cotton is one of the most significant crops produced in Pakistan. Cotton contributes to national economic development and for supporting the livelihoods of millions of farm families. Cotton has tremendous export potential, accounting for 55% of all foreign exchange incomes in Pakistan. Of the total farmers, 26% grow cotton in Pakistan, whereas 15% of the total cultivated area is devoted to cotton cultivation [9]. Approximately 65% of cotton is grown in Punjab province, which has arid conditions that are suitable for the crop. The remaining cotton is cultivated in Sindh, where the climatic conditions are relatively more humid [9]. Negligible areas in Khyber Pakhtunkhwa and Baluchistan are devoted to cotton. Cotton production contributes 4.5% of agriculture's value and 0.8% of the total GDP [10]. The area and production of selected countries using Bt varieties of cotton are presented in Figures 1 and 2. Figure 1 shows that the India, USA, Pakistan, China, Brazil and Australia are the leading countries in terms of cotton area devoted to Bt varieties of cotton [11]. Figure 2 shows that China, India, Pakistan, Brazil, Turkey and Uzbekistan were the top cotton-producing countries [12].

It is necessary to support cotton production, reduce the cost of production, improve quality, and implement policies to make farming profitable. Despite this, the agriculture sector has remained underdeveloped, and enhancing its performance is crucial for economic growth and poverty reduction. The present review is an integrated review to explore the cotton landscape in the world under prevailing conditions of Insect Pests and diseases. Effective management of insect pests is essential as they play a significant role in reducing the cotton yield. Over time, the insects' pests have gained resistance; this resistance is associated with the unwise application of chemicals. This review aimed to endorse accurate spraying techniques to manage the infestation of insect pests and conserve the ecosystem. This review was initiated from the (i) background of cotton production in the world, (ii) varietal development in cotton, (iii) problems (increasing susceptibility) in Bt varieties (iv) reliance on pesticides and (v) myths of pesticides applications followed by pesticides knowledge among farmers.

2. VARIETAL DEVELOPMENTS IN COTTON

2.1. Inception and Promotion of Bt Cotton

Conventional cotton was predominantly the critical choice of farmers before the inception of transgenic cotton. Over the period, traditional cotton was perceived as less effective for different reasons. Of the major reasons, low production was the foremost. Infestation of pests, especially armyworms, was intensive on conventional cotton, resulting in the high cost of production and exhibiting less production. Abro *et al*. [13] identified that cotton bollworms, mainly American, Pink, and Spotted bollworms, were the offensive pests, bringing around 30-40% yield loss. Farmers had to apply extensive chemicals to control the bollworms, which not only skyrocketed the cost of production but also accelerated the resistance of bollworms. PACRA [14] reported that during the fiscal year 2022, Pakistan imported pesticides worth USD ~201.7 mln, and ~69% of it was consumed on cotton crop only. In this context, conventional

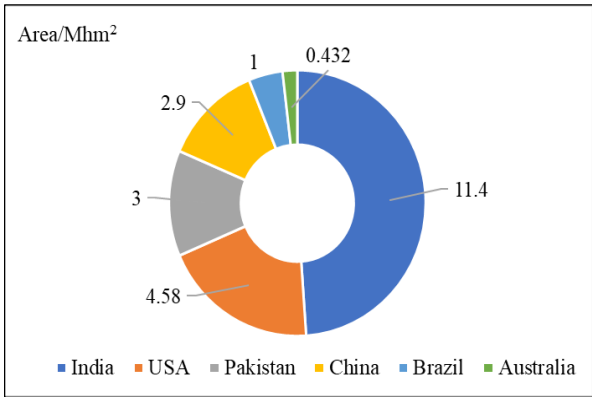

Fig. 1. Bt cotton cultivated area (Mh) in top cotton growing nations [11].

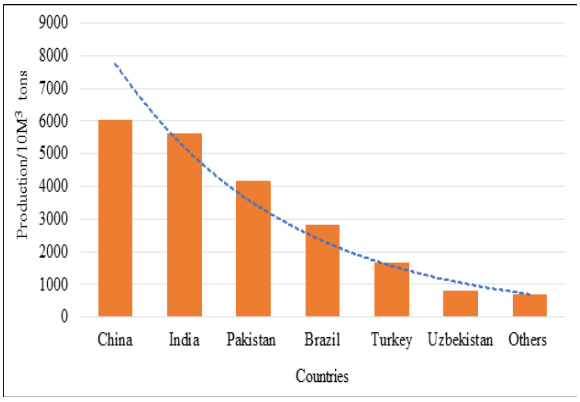

Fig. 2. Country-wise production of cotton in 2018-19 (1000 metric tons) [12].

cotton was deemed important for upgrading to new germplasms that are likely resistant to bollworms. The Pakistani government, in collaboration with the Center of Excellence in Molecular Biology (CEMB) and the National Institute for Biotechnology and Genetic Engineering (NIBGE), invested significant resources and human resources to develop genetically modified local varieties, specifically to tackle the issues in cotton production [15].

The first Bt variety of cotton was informally grown in Pakistan in 2002 [16]. However, the Bt variety obtained the final approval of the Government of Pakistan for cultivation in 2010. Contrary to Pakistan, the USA approved and commercialized the first Bt cotton variety in 1996 [17]. James [18] found that 12.1 million farmers had adopted GMO Genetically Modified Organisms) cotton, with the majority of them in China and India. In Pakistan, some private seed companies introduced the first Bt variety seed in the district Rahim Yar Khan [16]. The average yield of cotton in Pakistan was 0.5 t $ha^{-1,}$ considerably lower than the average yield achieved in China 9 t ha-1, indicating a wide yield gap in Pakistan [19]. The inception of the Bt variety was deemed important in bridging this yield gap of cotton prevailing in Pakistan [19]. The details of Bt and Non-Bt varieties approved in Pakistan from 2010-19 are tabulated in Table 1 [20].

2.2. Performance of Bt Varieties

Bt varieties were aimed at curtailing the undue application of pesticides and allowing growers to control insects and pests through minimum use of pesticides. Some benefits of Bt varieties are less attack of bollworms, minimum use of pesticides, high yield potential, and reduction in labour, which lure farmers to adopt Bt varieties over the non-Bt varieties [21]. Studies such as Qaim *et al*. [22], Bennett *et al*. [23], Subramanian and Qaim [24] and Kouser and Qaim [25] have confirmed that

Table 1. Bt and Non-Bt varieties approved in Pakistan from 2010-2019 (Adapted from Razzaq *et al.* [20]).

Sr. no.	Variety name	Year of release	Sr. no.	Variety name	Year of release
	Bt varieties		30	IUB-13	2015
1	IR-3701	2010	31	FH-LALAZAR	2015
2	Neelum-121	2010	32	BS-52	2015
3	FH-113	2010	33	BH-184	2015
4	Sitara-008	2010	34	Cyto-177	2015
5	MG-6	2010	35	AGC-999	2015
6	Ali Akbar-703	2010	36	MNH-988	2015
7	Ali Kabar-802	2010		**Non-Bt varieties**	
8	IR-1524	2010	37	Cyto-124	2015
9	GN Hybrid-2085	2010	38	NIAB-2008	2015
	Non-Bt varieties		39	GOMAL-105	2015
10	Sindh-1	2010		**Bt varieties**	
11	CRIS-342	2010	40	CIM-602	2016
12	NIA-Ufaq-08	2010		**Non-Bt varieties**	
13	Malmal	2010	41	NIAB-KIRAN	2016
	Bt varieties		42	CIM-620	2016
14	Tarzan-1	2012	43	CRIS-129	2016
15	MNH-886	2012	44	RJ-120	2016
16	NS-141	2012		**Bt varieties**	
17	FH-114	2012	45	IR-NIBGE-4	2017
18	IR-NIBGE-3	2012	46	IR-NIBGE-6	2017
19	CIM-598	2012		**Non-Bt varieties**	
20	Sitara 009	2012	47	RH-668	2018
21	A-One	2012	48	CIM-632	2018
	Non-Bt varieties		49	NIAB-1048	2018
22	CIM-573	2012	50	NIAB-545	2018
23	FH-941	2012	51	Crystal-12	2018
24	FH-942	2012	52	Sitara-15	2018
25	GS-1	2013	53	Sahara-150	2018
	Bt varieties		54	CIM-610	2018
26	AGC-777	2015	55	RH-662	2018
27	MM-58	2015	56	FH-152	2018
28	LEADER-1	2015	57	FH-490	2019
29	VH-305	2015			

with increased adoption of Bt cotton pesticide use decreased and cotton yield increased. As a result of the adoption of Bt varieties, not only was the use of pesticides reduced, but the cost of production also significantly declined, and the incidences of poisoning were reported to decrease [25]. USA [26], Mexico [27], South Africa [28], China [29], India [30] and Pakistan [21] have witnessed the benefits of Bt cotton, and its adoption was positive for the farmers for economic reasons as the yield was higher than the conventional cotton varieties [31]. Specifically, small farmers' yield increased due to Bt cotton adoption [32, 33]. Hofs *et al*. [34] reported that the economic returns of the Bt adoption were very bright, primarily due to a significant reduction in pyrethroid production. The overview of income benefits obtained from adopting Bt varieties is portrayed in Figure 3. This shows that India, China, USA, Pakistan, Brazil, Australia and Argentina were leading countries harvesting noteworthy income through genetically modified cotton [35].

3. PROBLEMS (INCREASING SUSCEPTIBILITY) IN BT VARIETIES

Bt varieties had resistance against the bollworms [13]. Despite its effectiveness, Bt cotton did not show high efficacy against insects that suck sap, such as whitefly, jassid, thrips, and mealybugs [36]. Dutt [37] reported a high infestation of mealybugs in Bt cotton varieties. Over time, Bt varieties are becoming vulnerable to pests and diseases, and their resistance is decreasing. Bt cotton contains a gene from the Bt bacterium that produces endotoxins harmful to certain Lepidopteran pests, including the Pink Bollworm [38]. However, the unforeseen

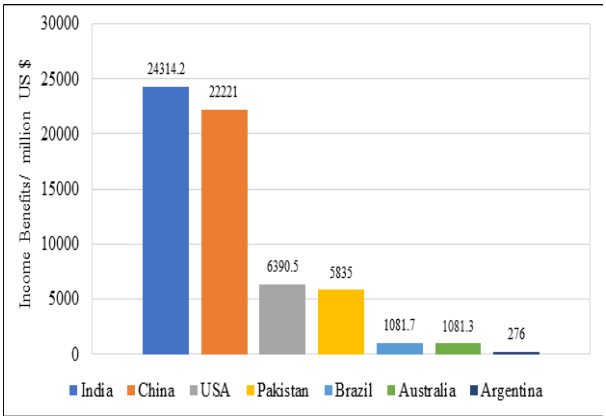

Fig. 3. Income benefits of genetically modified (GM) cotton farm in selected countries, 1996–2016 (million US$) [35].

return of key cotton pests that Bt was aimed to resist has augmented a new debate to rethink the resistance level of Bt cotton [39, 40]. Different pests attack Bt in various varieties, like different bollworms and whiteflies. In the subsequent section, debate is made about how bollworms, sucking pests, insects and viruses are influencing Bt varieties.

3.1. Bollworms

Bt varieties have resistance against the bollworms, which were more hazardous in lowering the production and quality of cotton. Karar *et al*. [41] admitted that the risk of bollworms was significantly reduced in cotton after the introduction of Bt varieties. However, over the period, the resistance in Bt varieties decreased. Rabelo *et al*. [42], Zhang *et al*. [43] and Tabashnik *et al*. [44] reported that the Bt resistance against the bollworms was decreasing. The attack of Pink Bollworms on Bt cotton is more often visible [41]. Pink Bollworm (PBW) infestation on Bt varieties surprised the growers, and this attack caused a severe loss in yield and income [45]. The results of Rajput *et al*. [46] were confirmatory that PBW caused a reduction in the yield and caused damage to cotton lint quality, producing yellow spots on the fibre. This infected lint got lower rates in the market and significantly lowered the farmers' income.

PBW was also found to be responsible for delaying the maturity of cotton [47]. Studies were evident that PBW attacked all varieties of cotton. For instance, Gutierrez *et al*. [48] identified that PBW damaged the cotton crop grown in rain-fed condition and damage was relatively more serious as compared to the cotton grown in irrigated conditions. This damage was associated with climatic variations. However, they questioned the use and quality of Bt cotton seed and the number and quality of insecticides applied, especially in rainfed cotton. The findings of Lu *et al.* [49] were different to the studies conducted in Pakistan and India, as they found that Bt cotton varieties were effectively capable of controlling the population of bollworms in China. Resistance management methods are employed to control bollworms, significantly to alleviate the host plants and improve the resistance in Bt varieties. Rajapakse and Walter [50] found more attacks of bollworms in the cotton field as many weeds were growing around the field. In addition to field cleaning practices, the cultivation

of natural refuge crops has been identified as a successful strategy to postpone the development of resistance to cotton bollworm [51]. It was recommended to grow non-Bt varieties alongside Bt varieties as a means of sufficiently delaying the development of bollworm resistance to Bt [52].

3.2. Cotton Leaf Curl Virus (CLCv)

The Gemini virus is responsible for causing Cotton Leaf Curl Disease (CLCv), which is a severe issue in cotton-producing regions worldwide. This virus spreads through the whitefly (*Bemisia tabaci*), and it can devastate the crop, leading to up to a 90% reduction in yield. This can also deteriorate the fibre quality [53]. The initial outbreak of CLCv was observed in Nigeria in 1912, and it has since spread to various cotton-producing countries, including the United States of America, Pakistan, India, and China [54]. Sattar *et al.* [55] argued that India and Pakistan were facing whitefly infestation in the cotton crop, resulting in losing the resistance. In 1989, CLCv was detected in the experimental fields of the Indian Agricultural Research Institute, New Delhi. Similarly, the existence of CLCv was later seen in Karnataka state, South India [56], and Northwest India in 1994 [57]. Specifically in Pakistan, the CLCv was first reported in Multan in 1967, and was perceived as a minor disease till 1987. However, it has spread over one million hectares of cotton area over the years as presented in Table 2 [58, 59]. CLCv damaged approximately 60% of the cotton crop in Multan in 1988, and the damage continues to occur each year. Pakistan and India have seen two epidemics of CLCv attack on cotton, causing a significant loss in production [55].

The incidences of CLCv are constantly changing over time, pointing towards the likely increase in yield decline [60]. CLCv grows in a suitable environment. Whitefly vectors can expedite the mode of spread [55]. The epidemiology of CLCv disease was related to abiotic factors like temperature and age of the plant [61]. Amrao *et al.* [62] found CLCv a very prominent disease. Around 70% of yield decline was witnessed subject to CLCv. The adverse impacts of the CLCv were more prominent on ginning out-turn and fibre fitness [63]. Nazeer *et al.* [64] stated that none of the varieties of *G. hirsutum* had resistance against the CLCv, although *G. Arboreum* has resistance against CLCv. In another study, Nawaz *et al.* [61] suggested changing the sowing times, cultural techniques, crop nutrition, buffer crops, whitefly

Table 2. Important landmarks in the history of CLCv. (Adapted from Rahman *et al*. [54]).

Year	History of CLCv
1912	• First report of CLCUD in Nigeria
1924	• Spread in Africa
1967	• CLCuD reported in Pakistan
1990	• First epidemic in Pakistan • Characterization of CLCuD initiated
1997-99	• Discovery of DNA alpha-satellite in 1999 • CIM-1100 was the first resistant variety followed by a number of varieties including CIM 448, CIM-496, NIBGE-2, FH-901, FH-1000, MNH-552, etc released in subsequent years
2001	• In 2001, CLCuBuV appeared in Pakistan • Beta-satellite discovered • DNA marker associated with resistance were identified
2005-10	• Antisense RNA, RNAi, etc has been utilized • Introgression breeding started • Release of tolerant cotton genotypes (NIBGE-115 and NN-3) • CLCuD reported in China
2012-13	• Dentification of asymptomatic cotton accession • Utilization in breeding as well as for DNA marker identification
2016	• Development of asymptomatic cotton lines • Identification of QTLs associated with diseases resistance

control and use of systemic chemicals followed by the seed treatment to manage the CLCv outbreak. Contrarily, late sowing of cotton, excessive use of nitrogenous fertilizer, and more attacks on insect pests exuberated the CLCv [65].

3.3. Sucking Insect Pests

Insects that suck sap, such as whitefly, thrips, and aphids, can be harmful to the health and productivity of cotton plants. Sucking insect pests have swapped the cotton bollworms. Since the inception of Bt varieties of cotton, about a 97% decrease in the chemical application was recorded. In the meantime, insecticides application jumped by 154% to control the sucking insect pests [66]. The overuse of insecticides had negative environmental consequences. Bt cotton varieties were not adequately resistant to insects that suck sap [67]. Therefore, continuously applying pesticides and insecticides is necessary to manage insect pests [34]. If pesticides are not appropriately applied, the population of insect pests multiplies, with significant inverse percussions on the crop [68].

Several studies like Abro *et al.* [13], Naveen *et al.* [69] and Sun *et al.* [70] augmented the higher infestation of sucking pests, including thrips, jassid, and whitefly, causing a significant decline in the cotton yield [71]. Jassid, thrip and Whitefly sucked the sap from the plant, turning the plant weak and ending in wilting and shedding of the leaves. These sucking pests have specific damage at the seedling stage of the crop and vegetative growth stage [13]. Whitefly further exaggerated the loss causing cotton leaf curl virus (CLCv) on cotton crops. India, one of the leading cotton producers, has reported a severe attack of whitefly on cotton during 2015-16 [69]. Sucking pests had a direct association with temperature and rainfall. Murtaza *et al.* [72] depicted that climate change had a significant impact on the population of sucking pests like Jassid. The findings of Harde *et al*. [73] confirmed that there was a positive correlation between the occurrence of sap-sucking pests such as whitefly, Jassid, Thrips, Mealybusg, and Aphid and the maximum temperature, minimum temperature, and maximum relative humidity. In another study, Shahid *et al.* [74] pointed out that the thrips and mites' population was high in June, August and September, augmenting the Jassid, whitefly and mealy bug population. This accentuates that with climate change, there are more chances of a high population of sucking pests. Therefore, with the increase in population, the chances of cotton yield losses will remain higher.

4. RESISTANCE DEVELOPMENT IN PESTS

Pertinent to increased resistance, the efficacy of Bt cotton varieties has declined [75]. Different factors contribute to the development of resistance to insect pests. Noncompliance with environmental protection agency regulations, inadequate production of high Bt endotoxins, repeated exposure to the same Bt endotoxins, and cross-resistance to multiple Bt endotoxins [76]. The variation in resistance of cotton armyworms and bollworms to insecticides was linked to the varying expression levels of Cry1Ac in the field conditions. This expression level was influenced by the plant's variety, age, and environmental factors [77, 78]. The resistance of insect pests was also found to increase due to insecticidal efficacy. The effectiveness of Bt varieties as insecticides was uneven due to the fluctuating expression of Bt protein throughout the cotton-growing season [79]. Insecticidal efficacy and ability were directly or indirectly predisposed by insect pests, disease intensity, rainfall amount, soil attributes, and apposite farm management. It can be deduced that an optimal environment was obligatory for Genetically Modified (GM) Cotton, which eventually reinforced the Bt gene expression. Huang *et al.* [76] specifically cited that in India, the development of resistance in pink bollworm (PBW) took seven years, which was attributed to the cultivation of unauthorized Bt cotton seeds. This resistance was associated with the low dose of Bt protein and non-compliance with the refuge strategy. Luo *et al.* [80] and Sethi and Dilawari [81] agreed that resistance in cotton insects and pests was due to the unwise application of pesticides on the crop. Sparks and Nauen [82] reported serious resistance in B. tabaci (whitefly), which is known for its enormous damage to the cotton crop in particular. The increased resistance in whiteflies has been reported in some countries [83, 84], and intensive pesticide use for insect and pest management inflated the infestation of whiteflies [85]. Ahmad *et al.* [63] reported a moderate resistance of whitefly against pyrethroids. It can be deduced that resistance to insect pests is increasing, and the unwise application of pesticides remains the critical reason.

4.1. Reliance on Pesticides Application

According to the report of Khan *et al.* [86], A total of 4875 kg of pesticide active ingredients were applied by farmers in the cotton belt of Punjab, Pakistan, per annum. The import of pesticides is also increasing each year [87]. Around 80% of all pesticides are used on cotton crops, while the remaining 20% are used on other crops like rice, sugarcane, maize, wheat, fruit orchards, and vegetables [87]. In the last 20 years, an expansion of 11.69% in the application of pesticides has been witnessed in Pakistan, and the total number of sprays per crop has reached more than 10 [88]. This extensive application is a point to ponder as human health is vulnerable to the impacts of chemicals, and insect pets have developed a high resistance level against the chemicals. No wonder the application of pesticides showed an increase in production by controlling the infestation of insect pests. From 1995 to 1998, an unexpected outbreak of insect pests caused a significant reduction in cotton yield from 849 to 230 kg/ha [89]. To combat this problem, the Government of Pakistan and pesticide manufacturers implemented a program involving increased pesticide use for monoculture crops. As a result, cotton production substantially increased from 2000 to 2001 [90]. The average yield in 1999-2000 reached 643 Kg/ha, from 494 Kg/ha, as recorded in 1997-98 and 1998-99. However, farmers in cotton-growing regions were applying 8 to 13 sprays per season and exceeding the recommended dosage to combat insect pests of cotton [91].

The extensive utilization of insecticides for managing whiteflies and other co-existing pests has resulted in a significant reduction of its natural enemies and the development of resistance to most of the conventional insecticides, thereby largely triggering whitefly outbreaks [63, 92]. According to the Central Cotton Research Institute (CCRI) in Multan, the two main cotton pests, the American bollworm and the whitefly, have developed resistance to commonly used pesticides [63, 92]. According to the Ministry of Food and Agriculture's report, there is evidence of a pesticide treadmill in Pakistan, as seen by the significant increase in applications of monocrotophos, cypermethrin, methamidophos, and dimethoate for controlling pests such as the American bollworm and whitefly. The application of monocrotophos has increased by 19 to 720 times, cypermethrin by 26 to 168-fold, methamidophos by 40 to 492-fold, and dimethoate by 104 to 725-fold to control these pests. Tariq *et al.* [91] indicated that before 1983, only a small percentage (5 to 10%) of cotton-growing regions in Punjab were treated with pesticides. However, this percentage increased to 100% by 1997. Overusing pesticides leads to insect pests developing resistance and poses a risk to farmers and community health. Excessive use of pesticides, such as spraying more than the recommended dose, in cotton fields poses a significant risk to field workers and pickers. It may result in unacceptable residue concentrations in cottonseed oil and cakes [90]. The discussion on the use of pesticides suggests that while their application has both positive and negative effects, the outcome depends largely on the spraying methods used by farmers. Further discussion on the various spraying techniques follows.

4.2. Myths of Spraying Techniques

Spraying techniques are of great worth in the wake of getting effective control over insect pests. In this regard, farmers use different spraying techniques. According to Tahir *et al.* [93], farmers typically use Knapsack and tractor-mounted boom sprayers to apply pesticides to crops in Pakistan. However, a significant issue with these sprayers is that the nozzle pressure is not consistently maintained during application, resulting in pesticide loss due to dribbling or drift. These problems increase production costs and contribute to environmental pollution and ecosystem imbalances. Damalas and Eleftherohorinos [94], most of the pesticides applied to the crops are wasted when they are used by farmers using improper techniques and nozzles. This wastage is an extra addition to the cost of production indeed.

Proper use of spraying techniques and nozzles greatly contributed to combatting insect pests. The type of nozzle, size, pressure on droplet size, and velocity are some aspects that farmers should consider palatably while spraying. The concerns made in this regard not only save the quantity of the pesticide but also curtail the cost of production [95]. Nozzles are classified based on droplet size and spray pattern [96]. Nozzles have three significant roles: breaking down the liquid into small droplets, dispersing these droplets in a specific pattern, and controlling the sprayer's release rate. Unfortunately, farmers have a poor

understanding of nozzle function and often use too much or too little pesticide, leading to ecological disruption and pesticide waste [97]. Due to untrained pesticide applicators and faulty spray equipment, many pesticides sprayed on crops reach non-target areas [97]. Excessive use of pesticides can result in the death of unintended organisms, leading to the potential resurgence of insect pests [98]. Insecticides have become less effective against certain cotton pests, such as Heliothis and whitefly, due to the inappropriate use of pesticides [99, 100]. Ejaz *et al.* [101] augmented that approximately 50% of the pesticides applied to various crops are wasted on non-targeted areas, leading to increased production costs and environmental degradation of the surrounding soil, water, and air. This is mainly due to ineffective spraying machines that cannot maintain the specified nozzle pressure, discharge, and height, which impacts spray pattern, droplet size, and uniformity. The size of droplets can also affect the spray coverage, and as droplet size decreases, the potential for drift increases [102]. Klein *et al.* [103] pointed out that boom sprayers' non-uniform nozzle tip output resulted in a misapplication of pesticides. The components of a nozzle include a nozzle body, a strainer, a replaceable nozzle tip, and a cap for securing it. Although farmers in Iran have excessively used chemical pesticides, there is little comprehension of their criteria for selecting and utilizing pesticides [104]. Ensure the use of pesticides correctly by paying attention to the pressure and the spraying situation before application [105]. Improper use of spraying techniques harnesses its damage to human health as well. While developing countries use only 20% of the world's total pesticide volume, they suffer from disproportionately high pesticide poisoning rates [106]. This pity situation spotlights a dire need to combat the issue of improper handling and use of pesticides in the wake of effective ecosystem conservation and escalated knowledge levels to combat the issue of improper use of pesticides. In this regard, knowledge regarding pesticide application is regarded as mandatory.

4.3. Dilemmas of Pesticide Knowledge

Among various factors behind the ineffective use of spraying techniques and wastage of resources, inadequate knowledge on the part of farmers is more prominent. Knowledge and information about the appropriate use of pesticides are regarded as key to the effective use of pesticides [107, 108]. Farmers in Pakistan usually lack the knowledge to select appropriate pesticides and effective spraying techniques as per their needs. Allahyari *et al.* [109] reported that farmers moderately understood precise spraying techniques. Most of the farmers preferred pesticides that were easy to access from dealers and used in the field [110]. Ejaz *et al.* [101] reported a lack of knowledge regarding the detrimental consequences of pesticide exposure during pesticide application. Various researchers such as Lekei [111], Ngowi [112], Karamidehkordi and Hashemi [113] have reported that farmers' limited education levels and inadequate training of pesticide management have resulted in their insufficient knowledge about the appropriate use of pesticides in suitable amounts. Farmers had limited adoption of recommendations for the safe use of pesticides due to inadequate technical awareness and knowledge [114]. Farmers rely on traditional sources to acquire information rather than contacting technical institutions like plant protection and agriculture extension departments. Khan and Iqbal [115] reported farmers' over-reliance on fellow farmers for information as 70% of farmers obtained guidance on the safe use of pesticides from their fellow farmers. Rehman *et al.* [116] reported that fellow farmers were reported to be a primary source of guidance for selecting and using pesticides, including information on their proper handling and usage. In another study, Mubushar *et al.* [114] revealed that farmers were not firmly following the advice and recommendations disseminated by the extension field staff regarding the safe use of pesticides. The over-reliance of farmers on fellow farmers to acquire information has already been reported by various research studies such as Ashraf *et al.* [117] and Akinnagbe *et al.* [118].

5. IMPLICATIONS FOR THE WISE APPLICATION OF PESTICIDES AND INSECT PEST MANAGEMENT

Achieving higher production is the foremost concern for farmers and state institutions. For this reason, farmers implement whatever possible measure is needed. Among various options, the application of pesticides, insecticides and fungicides is perceived prominent as an infestation of insects, pests and diseases on crops not only significantly lower the level of production but also deteriorates the quality of produce. Tariq *et al.* [91] reported that a hefty pest

attack during 1995-1998 reduced cotton production from 849 kg/ha to 230 kg/ha. Mealybug infestations in Pakistan was expected to decrease cotton yield by 1.3 million bales [119]. Mallah *et al.* [120] and Abbas *et al*. [121] reported a 20-40% loss in cotton production due to different insects and pets of the cotton. Around 24% of cotton production loss was due to jassid [20].

The implications for applying pesticides and insect pest management in cotton crop cultivation are paramount for ensuring sustainable and productive agriculture. Cotton, a major cash crop, is susceptible to various insect pests that can significantly impact yields if uncontrolled. Wise application of pesticides involves a judicious use of chemical agents, considering factors such as pest species, developmental stages, and potential ecological consequences. Wise application of pesticides in the cotton crop can reduce weed-related yield reduction by 50 to 85% and increase production costs by addressing labour shortages and labour wages [122]. Although farmers use various pest control methods to increase production and reduce yield loss, no single method of controlling insect pests is considered sufficient to achieve the desired production level. Using chemicals to protect crops is beneficial and an essential component of integrated pest management [123]. The integration of control measures can increase production significantly. If cotton pests are effectively controlled, the yield can be increased by 200-300 kg per hectare [124]. The government of Pakistan and pesticide manufacturers launched a program aimed at increasing the use of pesticides for monoculture crops. As a result, cotton production experienced a significant boost from 2000 to 2001 [89].

Integrated Pest Management (IPM) strategies, encompassing biological, cultural, and chemical control methods, emerge as a holistic approach to curb pest populations while minimizing environmental impact. Adopting IPM addresses immediate pest concerns and contributes to long-term pest resistance management, preserving the efficacy of available pesticides. Integrated pest management (IPM) in cotton crops can lead to long-lasting, economical, and eco-friendly benefits [125]. In a study, Kranthi and Russell [126] revealed that Integrated Pest Management (IPM) programs, using naturally occurring pest control components, result in favourable ecological, sociological, and environmental consequences for the cotton crop. The implications for the wise application of pesticides extend beyond pest control to broader environmental and economic dimensions. Overreliance on certain pesticides can lead to the development of resistant pest populations, necessitating a rotation of active ingredients to maintain efficacy. Moreover, indiscriminate pesticide use can adversely affect non-target organisms, soil health, and water quality [94]. A wise and informed approach involves selecting pesticides with lower environmental impact, adhering to recommended application rates, and incorporating non-chemical pest management methods whenever possible [127, 128]. Economically, the judicious use of pesticides minimizes production costs and conserves resources. By investing in research and extension services that promote wise pesticide application and integrated pest management, the agricultural sector can strike a balance between effective pest control, environmental stewardship, and economic sustainability in cotton cultivation [129]. Matteson [130] stated that Integrated pest management (IPM) extension education encourages wise pesticide use and empowers farmers to manage a healthy paddy ecosystem, promoting ecological balance in rice paddies. Improved agricultural extension services, such as the PlantWise program in China, lead to more sustainable pest management by increasing recommendations for biological control, pest monitoring, and cultural control [131]. Integrated pest management training in school extension programs effectively achieves sustainable agriculture [132]. Similarly, the Insecticide Resistance Management (IRM) program in India significantly improved farmers' knowledge in identifying insect pests, proper use of insecticides, and timely sowing of the cotton crop, but limited their understanding of cultural practices.

6. CONCLUSIONS AND RECOMMENDATIONS

Insect pests and diseases are key factors lowering the cotton yield. Insect pests in cotton crops are managed by applying various pesticides, which not only increase the cost of production but also contribute to environmental degradation. Over the years, insect pests have attained resistance against pesticides; even the resistance of the Bt gene has decreased being susceptible to bollworms (i.e. Pink Bollworm) and other sucking pests (i.e.,

whitefly). The unwise application of pesticides on cotton crops is the foremost reason for increasing resistance to insect pests. Cotton growers are usually unaware of the technical side of the spraying. Using traditional spraying methods was the ineffective and injudicious application of pesticides that caused environmental pollution. Farmers are deficient in technical knowledge about the safe application of pesticides. The deficiency of technical knowledge is also associated with reliance on traditional information sources (i.e., fellow farmers). Therefore, it is essential to improve farmers' technical knowledge of spraying techniques and curtail the cost of production. Helping farmers in pesticide selection, controlling overdosing/underdosing, and conserving the ecosystem, environment, soil and water should be priorities. Advisory service-providing institutions should technically guide the farmers regarding the safe use of pesticides. Developing trained and technically sound farmers who can serve as an information source for other farmers seems another vital avenue to consider. Moreover, there is a need for the development of sucking pest-resistant cotton varieties followed by the implementation of integrated pest management techniques.

6.1. Direction for Future Research

Based on this critical review, the following future research directions are proposed;

1. Future research could explore and evaluate alternative pest management strategies that reduce reliance on traditional spraying techniques. This might involve the assessment of biological control methods, such as the introduction of natural predators or the use of microbial agents to control sucking pests.
2. Research could focus on integrating precision farming technologies to optimize pesticide application in cotton fields. Precision agriculture tools, such as drone-based monitoring, satellite imaging, and sensor technologies, can potentially target pest-infested areas more precisely.
3. Understanding the factors influencing farmers' decision-making regarding pesticide use is crucial. Future research could delve into behavioural studies to explore the motivations, knowledge gaps, and perceptions contributing to unwise spraying techniques. Developing targeted educational interventions and extension programs to enhance farmers' understanding of pest management practices, including the consequences of unwise spraying, could be an effective strategy to address the issue.
4. To comprehensively address resistance development in sucking pests, a longitudinal study could be conducted to monitor resistance patterns over an extended period. This research would involve regular sampling and analysis of pest populations in cotton fields to track changes in resistance levels. Understanding the dynamics of resistance development over time could inform the development of more sustainable pest management strategies.

7. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 149-157 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)919

Pakistan Academy of Sciences

Research Article

Assessment of Knee Osteoarthritis Severity using New Multifactorial Scale (KHIUS) in Northwest Syria

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Abstract: The present study aimed to identify the risk factors associated with knee osteoarthritis (OA) in Northwest Syria and to evaluate the reliability of our newly proposed Khatib-Khaled Idlib University Scale (KHIUS) in assessing knee OA severity. The study enrolled 101 patients with knee OA, diagnosed through X-ray at the orthopedic clinic. The Kellgren and Lawrence classification was employed to determine the X-ray knee OA grades. The erythrocyte sedimentation rate (ESR) value was obtained as a biomarker after excluding rheumatoid arthritis, other inflammatory diseases, and malignant tumors. The risk factors of knee OA assessed in our study included age, gender, BMI, and physical activity. Each patient completed the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) questionnaire and KHIUS to evaluate knee OA severity. Correlation coefficients of two scales, i.e., WOMAC and X-ray knee OA grading as well as KHIUS and X-ray knee OA grading, were determined. The mean age of the patients was 52.84 ± 9.74 years (age range 25-80 years). Most patients had low daily activity levels, and the left knee was the most affected. In our study, the correlation coefficient between WOMAC and KHIUS was strong (R: 80.3%, $P < 0.01$). The correlation coefficient between X-ray KL knee OA grades and WOMAC was moderate (R: 50.9%, $P < 0.01$), whereas the correlation coefficient between X-ray KL knee OA grades and KHIUS was comparatively stronger (R: 75.7%, $P < 0.01$). KHIUS can be a reliable scale to assess knee OA severity and to guide the method of treatment by orthopedic surgeons. In addition, KHIUS is more closely related to X-ray KL knee OA grading than other clinical scales.

Keywords: Knee Osteoarthritis, KHIUS, Risk Factors, ESR, Biomarker, WOMAC.

1. INTRODUCTION

According to the World Health Organization (WHO), Knee Osteoarthritis (KO) is a prevalent condition worldwide. The global incidence rate of this disease is 5% among the adult population over the age of 18 years, with the rate increasing to 10% in males and 14% in females aged between 50 and 69 years old in patients with hip and knee OA [1]. KO is the 4th leading cause of disability in Asia [2], with a confirmed correlation between incidence and income. In high-income regions, there are 358 cases per 100,000 individuals compared to 75 cases per 100,000 individuals in low-income regions. Likewise, in the USA, statistics indicate a disparity in the occurrence of Knee OA between African and White Americans [3]. Studies have shown that a significant number of individuals with radiographically confirmed OA do not experience symptoms. Therefore, the prevalence of radiographic knee OA is believed to be higher than symptomatic knee OA. Knee pain serves as a vague indicator of radiographic knee osteoarthritis, which is somewhat influenced by the extent of radiographic involvement. Similarly, radiographic knee OA provides an uncertain indication of the likelihood of experiencing knee pain or disability. Many individuals with radiographic knee OA do not exhibit symptoms, while conversely, many patients with knee pain suggestive of OA lack radiologic findings. For instance, in South Korea, the prevalence of radiographic knee OA was

Received: June 2023; Revised: March 2024; Accepted: June 2024
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reported to be 21.1% in males and 43.8% in females, while the prevalence of symptomatic knee OA was 4.4% in males and 19.2% in females [4]. Likewise, evidence from a Japanese population-based cohort study indicated a weak association between the symptoms of knee OA and radiographic findings, and vice versa [5]. According to a systematic literature review, 15-76% of individuals with knee pain showed radiographic OA, while 15-81% of those with radiographic knee OA experienced pain [6].

The incidence of OA is strongly associated with aging, as advanced glycation end products accumulate in the cartilage matrix and contribute to cartilage fragility. This process also stimulates the innate immune system in the synovial membrane, leading to the production of pro-inflammatory mediators such as cytokines, which can further contribute to the development and progression of knee OA [7]. In addition, the aging of chondrocytes can lead to a decrease in the production of growth factors such as insulin-like growth factor and transforming growth factor-β, which can further exacerbate knee OA [8]. The incidence of knee OA is higher in females due to the loss of estrogen after menopause, which affects the production of cartilaginous matrix proteins and stimulates the destructive activity of articular cartilage [9]. Obesity is another important factor that triggers OA pathogenesis and contributes to its progression. Obesity has both mechanical effects on weight-bearing joints such as the knee and hip and systemic effects on OA occurrence by releasing adipokines that lead to release of pro-inflammatory cytokines such as IL1β and TNFα, thereby inhibiting the production of collagen II and aggrecan from chondrocytes [10, 11]. However, it is worth to mention that all obese individuals do not develop OA. The most likely explanation for this disparity could be due to other factors such as genetics, joint alignment, and physical activity levels, which may also contribute to the pathogenesis and progression of knee OA. As such, physical activity has shown both positive and negative effects on knee OA, while the atrophy of muscular mass surrounding the knee joint increases the incidence rate of knee OA [12, 13]. Workers in certain occupations such as builders, farmers, firefighters, fishermen, foresters, and miners have also been associated with an increased incidences of knee OA [14]. Various scales are used to assess knee function, including the International Knee Documentation Committee (IKDC), Knee injury and Osteoarthritis Outcome Score (KOOS), Lysholm Knee Scoring Scale, Oxford Knee Score (OKS), and Western Ontario and McMaster Universities Osteoarthritis index (WOMAC) [15-19]. While these scales provide insight into knee OA severity, these rely solely on patient responses to classify the condition. In other words, the current knee assessment criteria used in the above-mentioned scales, including pain severity and joint disability scales, have limitations that affect the accuracy of the assessment due to the fact that these scales depend completely on patient-reported answers, which can vary over time despite the same level of joint degeneration. On the other hand, relying exclusively on X-ray findings in OA patients may not be sufficient, because patients with advanced osteoarthritis on X-ray may have mild or moderate pain and disability, while those with mild osteoarthritis on X-ray might be suffering from severe pain and disability. Therefore, clinical scales alone cannot be used to assessment of knee osteoarthritis. In the present study, we have proposed a new scale called "Khatib-Khaled Idlib University Scale" (KHIUS) which does not solely rely on the patient questionnaire but additionally utilizes clinical information based on radiographic finding, ESR values and physical examination. The goal of this study was to identify the risk factors associated with knee osteoarthritis (OA) in Northwest Syria and to assess KHIUS that we have proposed and determine its reliability in evaluating the severity of knee OA.

2. MATERIALS AND METHODS

The present study included 101 patients diagnosed with knee osteoarthritis based on simple X-Ray at the Orthopedic Clinic in Idlib University Hospital. The severity of osteoarthritic X-Ray grading was determined using the Kellgren and Lawrence (KL) classification, while the first-hour ESR value was also obtained.

Firstly, we collected personal information and risk factors of knee OA, including age, gender, Body Mass Index (BMI), and physical activity. Then, each patient, who suffered knee OA, was assessed using both WOMAC questionnaire and KHIUS criteria to determine knee OA severity.

WOMAC consists of 24 items divided into three subscales: pain, stiffness and disability. Each subscale consists of several items and points are

given for each item based on the patient's answer. The points are graded as follows: none (0), mild (1), moderate (2), severe (3) and extremely (4). The minimum total value in WOMAC is 0 while the maximum is 96.

KHIUS (Khatib-Khaled Idlib University Scale) includes 16 items divided into the following four subscales:

(A) Pain and disability: This subscale consist of eight items (Table 1), such as pain severity, pain and disability when standing, walking, getting up from a sitting position, at night, when ascending and descending stairs, in the bathroom, during housework, and during work and activity outside the house. Points are given for each item based on the patient's answer, and the points are graded as follows: none (1), mild (2), moderate (3), and severe (4). The minimum value in this group is 8, and the maximum is 32.

(B) Physical Examination: Physical examination is a crucial part of the knee OA assessment and includes six items, as shown in Table 2, including knee range of motion, swelling, patellar pressure test, joint line tenderness, flexion contracture, and knee stability. The minimum score in this group is 6 and the maximum is 24. Knee instability is an important factor in accelerating joint degeneration. Therefore, KHIUS awards 4 points when any form of knee instability is present.

(C) X-Ray grading: X-Ray grading, based on the KL classification, is divided into four grades, and because of its significance in the final assessment of knee OA, KHIUS assigns 2 points for KL grade I, 4 points for KL grade II, 8 points for KL grade III, and 12 points for KL grade IV, as shown in Table 3.

(D) Elevated ESR: Abnormally high inflammatory markers such as ESR have been shown to be associated with knee OA. Thus, KHIUS assigns 4 points when ESR is below 30 and 8 points when ESR is above 30 (after excluding other inflammatory diseases, malignant tumors, and rheumatoid arthritis), as shown in Table 3. The minimum value of KHIUS is 20 and the maximum value is 76. The knee OA severity grades based on KHIUS are presented in Table 4.

2.1. Statistical analysis

The statistical analysis was performed using the SPSS-25 software. Descriptive statistics were used. Nonparametric Mann-Whitney U-test and

Table 1. Variables of pain and disability group in KHIUS.

Pain and disability	none	mild	moderate	severe
Pain severity	1	2	3	4
standing	1	2	3	4
walking	1	2	3	4
Getting up from sitting position	1	2	3	4
At night	1	2	3	4
Housework	1	2	3	4
Outside house activities	1	2	3	4
Ascending and descending stairs	1	2	3	4

Table 2. Parameters of physical examination group in KHIUS.

Physical examination	none	mild	moderate	severe
Swelling	1	2	3	4
Joint line tenderness	1	2	3	4
Patellar pressure test	1	2	3	4
Flexion contracture	1	2	3	4
Range of motion	140-160	120-140	100-120	< 100
	1	2	3	4
Knee stability	normal		instability	
	1		4	

Table 3. Variables of X-Ray findings and grading as well as ESR analysis in KHIUS.

X-Ray grading			
Grade I	Grade II	Grade III	Grade IV
2	4	8	12
ESR analysis			
Less than 30		Equal or More than 30	
4		8	

Table 4. Knee OA severity grades according to KHIUS.

Knee OA grades	**KHIUS points**
Grade I: mild knee OA	20-33 points
Grade II: mild to moderate knee OA	34-47 points
Grade III: moderate to severe knee OA	48-61 points
Grade IV: severe knee OA	62-76 points

parametric Student's t-test were used to compare the variables. Correlations between WOMAC and KHIUS were tested by Pearson correlation. Spearman rank analysis was used to investigate correlations between WOMAC and KL-knee OA grading, and between KHIUS and KL-knee OA grading to determine the most closely scale to radiographic grades of knee osteoarthritis.

3. RESULTS

Table 5 shows that females in the sample had a significantly higher mean BMI value compared to males ($P < 0.01$), as the BMI values were divided into ranges and analyzed for their relationship with gender. In addition, the majority of cases (78%) were females. Females exhibit a higher susceptibility to developing OA [20]. This increased vulnerability in women may stem from various factors, such as thinner cartilage, a predisposition to varus malalignment, joint instability, and uneven mechanical loading [21]. Other factors include the loss of estrogen after menopause and the prevalence of obesity among women in northwest Syria. The mean age of the samples was 52.84 ± 9.74 years, with the age range of the participants from 25 to 80 years (Table 5).

The statistical data indicated that grade II left knee OA was the most prevalent among the participants as per KL classification. Furthermore, it was observed that most patients had mild daily

Table 5. Statistical analysis of age and BMI (body-mass index) groups.

	Males (*n* = 22)	**Females (*n* = 79)**	**P-Value**
BMI (Mean SD)	28.91 ± 4.87	35.11 ± 6.11	0.0001
	BMI		
BMI (kg\m²)	**Males**	**Females**	**Frequency/ percent**
20-24.99	6	3	9 (8.9%)
25-29.99	7	16	23(22.8%)
30-34.99	6	20	26 (25.7%)
35-39.99	3	25	28 (27.7%)
40 and more	0	15	15 (14.9%)
	Age		
Age (years)	**Frequency**		**Percent**
Equal and less 30	1		1%
31 – 40	10		9.9%
41 – 50	32		31.7%
51 – 60	35		34.7%
>60	23		22.8%

activity levels (see Table 6). The mean value of WOMAC in sample was 42.43 ± 8.18, total WOMAC values correlated with age (r = 0.35; p < 0.001) and BMI (r = 0.266; p = 0.007). Women and men had significantly different disease severity scores: 43.49 ± 7.77 and 38.59 ± 8.66, respectively (p = 0.023). The mean value of KHIUS was 52.27 ± 7, total KHIUS values correlated with age (r = 0.50; p <0.001) and BMI (r = 0.28; p = 0.004). Women and men had no significantly different disease severity scores: 52.92 ± 6.36 and 49.95 ± 8.7, respectively (p = 0.078). There was strong correlation (r = 80.3%, P < 0.001) between WOMAC and KHIUS (Table 7), thus suggesting that the KHIUS has construct validity for assessing the severity of knee OA (Figure 1). Additionally, the correlation coefficient between X-ray knee OA grading based on the KL classification and WOMAC in our sample was moderate (R: 50.9%, P < 0.001), while the correlation coefficient between X-ray KL knee OA grading and KHIUS was stronger (R: 75.7%, P < 0.001) (Table 7 and Figure 2).

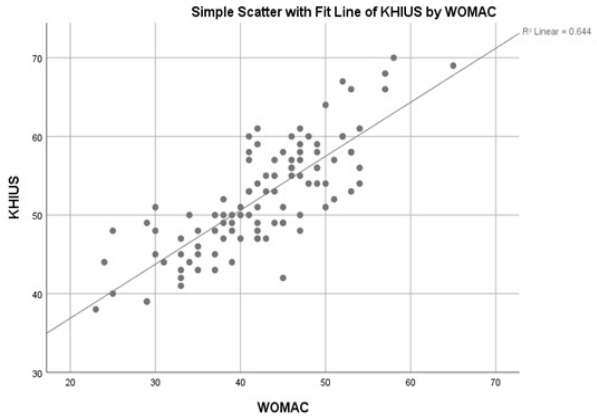

Fig. 1. Scatter plot of correlation of WOMAC to KHIUS.

4. DISCUSSION

The WOMAC score is a widely used clinical scale for evaluating the severity of knee and hip osteoarthritis on a global scale. Its reliability and validity have been established through numerous studies conducted worldwide, and it has been translated into multiple languages. According to previous research, the Cronbach's alphas for the WOMAC and Lequesne subscales ranged from 0.78-0.95 and 0.51-0.85 for hip OA, and 0.78-0.94 and 0.61-0.71 for knee OA, respectively [22]. Additionally, another study found that the test-retest reliability of all three WOMAC subscales (pain, stiffness, and physical function) was satisfactory, with ICCs of 0.86, 0.68, and 0.89, respectively [23]. The WOMAC score is extensively utilized for the pre- and post-evaluation of various therapeutic and surgical procedures [24, 25]. It involves asking patients a series of questions to assess the severity of pain, stiffness, and functional disability [26]. The WOMAC score is a subjective scale [27], and its effectiveness depends on the patient's cultural understanding of the questions asked. Cultural differences across the globe result

Table 6. Additional statistics.

		Frequency	Percentage
Daily activities	mild	72	71.3%
	moderate	26	25.7%
	high	3	3%
	total	101	100%
X-Ray grading according to Kellgren and Lawrence classification	Grade I	2	2%
	Grade II	49	48.5%
	Grade III	32	31.7%
	Grade IV	18	17.8%
	total	101	100%
Knee side affected	Right	29	28.7%
	Left	48	47.5%
	Both	24	23.8%
	total	101	100%

Table 7. Relationship between X-Ray grades with WOMAC and KHIUS.

		WOMAC (n = 101)	KHIUS (n = 101)
X-Ray grades (n=101)	r	0.509	0.757
	p	0.0001	0.0001
KHIUS	r	0.803	
	p	0.0001	

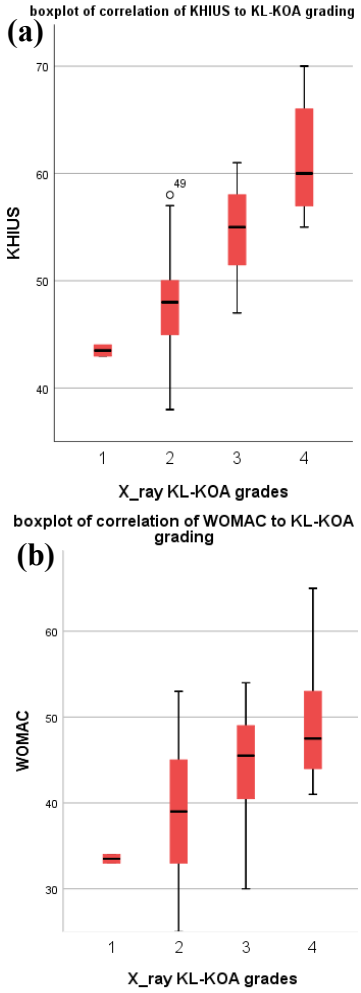

Fig. 2. Boxplots of correlation of (a) KHIUS and (b) WOMAC to KL knee osteoarthritis grades.

in varying responses to the questions asked which could potentially affect the accuracy of the score. In conflict areas, many patients may struggle to provide accurate responses due to their living conditions. For instance, people living in areas of protracted conflict do not have the luxury to sleep on beds. Rather, they sleep on the floor, thus making it impossible for them to answer questions related to getting up from a bed. Additionally, many people worldwide do not have access to cars, and therefore, those patients may not be able to accurately describe any difficulties they may face in using the car.

The WOMAC physical function subscale possesses a potential drawback due to its lack of clear distinction between the concepts of pain and function [28]. The WOMAC score is used to express the level of pain and disability experienced by patients with knee OA; however, this score may not always correlate with the radiological degree of the disease [29]. For instance, a low WOMAC score in some patients indicates mild degenerative disease, while radiographic grades in those patients indicates severe knee OA, hence, terms such as symptomatic knee osteoarthritis, radiographic knee osteoarthritis, and symptomatic radiographic knee osteoarthritis are widely used around the world [4]. To improve the accuracy of knee assessment, clinical data from physical examination such as range of motion, muscular atrophy, joint line tenderness, patellar tests, and knee stability should be included in the assessment. Moreover, the assessment should also include radiographic grades and the measurement of inflammatory markers, such as ESR, which have been shown to be important in the occurrence and progression of knee OA. Although rheumatoid arthritis (RA) is recognized as a severe inflammatory condition with elevated levels of ESR and CRP [30], OA has traditionally been viewed differently. It was believed that OA lacked significant inflammation, and therefore, serum markers of inflammation were not typically elevated in OA patients. However, recent studies have challenged this notion, revealing elevated levels of inflammatory markers, such as ESR and CRP, in individuals with OA. Studies have found a positive correlation between ESR levels and the severity of pain, stiffness, and functional disability [31, 32].

In this paper, we are proposing the KHIUS score to evaluate the severity of knee OA, which includes both subjective and objective measures. The subjective component assesses the patient's general daily activities without overly complex or repetitive questions, while the objective component includes physical examination data, radiographic

findings (based on the KL classification), and laboratory data (first-hour ESR value). The KHIUS score has a maximum value of 76, with 32 points allocated to the subjective component and 44 points to the objective component.

Of course, patients alone cannot determine their total KHIUS score since they can only use the subjective component to answer questions about their daily activities while they cannot determine the objective (clinical) component of data without the help of a clinician. However, orthopedic surgeons can use the entire KHIUS score to determine the severity of knee OA in their patients. By incorporating both subjective and objective measures, including patients' daily activities, physical examination data, radiographic findings, and laboratory data (such as ESR value), the KHIUS score offers orthopedic surgeons with valuable insights into the extent of knee OA and its impact on patients' functional status. This comprehensive evaluation enables clinicians to tailor treatment strategies to individual patients' needs, guiding decisions regarding the type and intensity of interventions required to manage knee OA effectively. Higher KHIUS scores may indicate more advanced disease and a greater need for aggressive interventions, such as surgical procedures. Conversely, lower KHIUS scores may suggest less severe disease and may prompt conservative management strategies, such as physical therapy or lifestyle modifications.

KHIUS score has its limitations. It may not be suitable for test-retest evaluation after treatment of knee OA due to the potential changes in objective data which comprises of radiographic findings, ESR value and physical examination findings. Therefore, the WOMAC score remains the most widely used method for test-retest evaluation after treatment in knee OA patients, while the KHIUS score may be considered the best method for determining the severity of knee OA.

Potential sources of bias or confounding factors that may affect the accuracy of the KHIUS score include its subjective nature, influenced by patients' interpretations and cultural differences, particularly in conflict areas or regions with diverse living conditions. Additionally, the reliance on clinicians for objective data introduces variability in scoring, limiting patient autonomy and potentially leading to subjective assessments. Measurement limitations, such as variations in imaging quality and inflammatory marker levels, can further impact score accuracy. Addressing these factors is crucial to enhance the reliability of the KHIUS score in assessing knee osteoarthritis severity and guiding treatment decisions effectively.

5. CONCLUSIONS

The Khatib-Khaled Idlib University Scale (KHIUS) was found to be closer to radiographic grades, indicating that it can be relied upon to assess knee OA severity and guide the method of treatment. Patients with knee OA can use clinical scales (such as WOMAC) to assess themselves, while orthopedic surgeons can use KHIUS after a complete examination and adding X-ray and laboratory test data. Therefore, KHIUS may serve as a reliable tool to assess knee OA severity in order to guide the method of treatment for patients with knee osteoarthritis. While our study utilized cross-sectional design to assess knee osteoarthritis severity using the WOMAC score, future research needs longitudinal or intervention studies for stronger causality evidence, providing insights into disease progression and interventions' impact for better prevention and treatment strategies.

6. ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent was obtained from the patients. The study was approved by the Idlib University Research Ethics Committee. Patient privacy and data confidentiality were maintained in accordance with the Declaration of Helsinki.

7. ACKNOWLEDGEMENTS

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

8. CONFLICT OF INTEREST

The authors declare no conflict of interest.

9. DECLARATION

We hereby declare that: (i) the results are original; (ii) the same material is neither published nor under

consideration elsewhere; (iii) approval of all authors have been obtained; and (iv) in case the article is accepted for publication, its copyright will be assigned to Pakistan Academy of Sciences.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 159-169 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)925

Pakistan Academy of Sciences

Research Article

Improving Soil Quality and Yield of Intercropping-System Crops in a Dry Land Area through Plant Growth Promoting Rhizobacteria Application Frequency

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Abstract: The future of agriculture is prone to choose technology that can enhance the quality of the resources to support the sustainability of food production. Plant Growth Promoting Rhizobacteria (PGPR) is a reliable technology for future agriculture as it is environment-friendly, and able to optimize resource utilization and decrease external input. This research aimed to analyze the effect of PGPR (*Pseudomonas fluorescens* + *Bacillus polymyxa*) application frequency on chemical soil properties, a yield of an intercropping system in dry land, the in-between correlation of the parameters, and to determine the best PGPR application frequency. Randomized Complete Block Design (RCBD) was used in this research to put the treatment in the experimental unit properly. The treatments consisted of i) one-time application of PGPR at the planting time, ii) twice application of PGPR at the planting time and 15 Days After Planting (DAP), iii) three times application of PGPR at the planting time, 15 DAP and 30 DAP, iv) without application of PGPR as control. The results showed that PGPR application frequency improved chemical soil properties, yield, and total by-products as livestock feed. The activity of soil enzymes, nitrogenase, and phosphatase, was enhanced compared to the control. The application of PGPR in dryland areas is recommended to maintain soil fertility and support sustainable intercropping crop production. Further studies are needed to conduct mixed farming between agriculture, animal husbandry, clean energy (biogas), and organic fertilizer (residue from the biogas digester).

Keywords: Arid Land, Bio Fertilizer, Environment-Friendly Technology, Mixed Farming, Multiple Cropping Practice, Sustainability Food Production.

1. INTRODUCTION

Agriculture in the future will face a significant challenge: providing proper food for the increasing world population. In 2050, the world population is projected to reach 9.7×10^9 [1]. Consequently, food production must be improved to cover the future population. Dryland can be an alternative source of food production as it has 40 % of the total land in the world [2]. The ecosystem in dry land has been proven to contribute to world development and promise for the future. However, converting dry land to agriculture has many obstacles, such as limited water availability, easily eroded soil, low organic matter content, and low nutrient content [3]. This condition implies that cultivating crops on this land reasonably needs a strategy to maintain sustainable resources and production. Sustainability is a demand for agriculture in the future [4], and conservation-based agriculture is more suitable to be developed [5]. In addition, apart from the land resource, other constraints of cultivation in dry land are narrow land tenure and a low number of educated farmers. This situation will further suppress sustainable food production. From the mentioned constraints, soil fertility and water availability are the main limiting factors for dryland food production. The limited water availability in dryland causes low

Received: June 2023; Revised: April 2024; Accepted: June 2024
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soil moisture, affecting soil microorganisms' life [6, 7]. An insufficient amount of soil moisture also affects both the low population and low activity of microorganisms [8]. In contrast, the population of microorganisms in dry land is an important factor as a bio-indicator for soil fertility [9, 10]. A high population of microorganisms indicates better soil fertility. To support the growth and production of crops in dry land, the population of beneficial microorganisms in the soil needs to be increased, especially plant growth promoting rhizobacteria (PGPR) [11]. These bacteria can provide available nutrients for crops [12, 13]. In addition, PGPR is also capable of producing phytohormone [14–17] and acting as a biocontrol [18–20]. Under environmental stress, PGPR helps plants overcome these unfavorable conditions to survive [6, 21, 22]. The role of PGPR can increase the growth potential of crops cultivated in unfavorable lands, such as dry land.

Another study reported that rhizobacteria *Pseudomonas flourescens* (Trevisan) Migula 1895; and *Bacillus* sp. could dissolve phosphate [23]. Moreover, these bacteria can synthesize plant growth hormone, namely indole acetic acid (IAA) [24]. *P. flourescens* and *Paenibacillus polymyxa* (Prazmowski 1880) can reduce salt stress's effect on the shoot and root growth of barley (*Hordeum vulgare* L.) [25]. Applying *P. fluorescence* as a biofertilizer in banana [*Musa* (*genus*)] nurseries resulted in colonized roots and encouraged the growth of banana seedlings [26].

There are several studies of PGPR applications in intercropping systems; however, they only compare PGPR inoculation and without PGPR [27, 28]. However, the effectiveness of PGPR application frequency in improving soil quality and yield in the intercropping system has not been widely reported. PGPR application in the intercropping system is an attempt to increase the availability of nutrients in the soil for crops. The availability of sufficient nutrients can reduce competition between plants, affecting the production of intercropping crops per unit of land area. The PGPR population in the rooting area needs to be maintained to still provide nutrients for plants in sufficient quantities, especially during the growth period of the plants. These reasons are the underlying reasons why PGPR application frequency should be taken into account. This study aimed to analyze the effect of PGPR application frequency on improving soil chemical properties, yield, and a total by-product as livestock feed in an intercropping system on dry land.

2. MATERIALS AND METHODS

2.1. Location

The experiment was conducted on dry land on Poteran island, Sumenep regency, Indonesia with a dry climate (Table 1) and an 8 m above sea level, from January to April 2019. This location is located at S 7°03'57.2832", E 113°56'31.7076". The soil type on this island is categorized as the Mediterranean, with soil temperature regimes as hot (is hyperthermic). Chemical soil properties on the site (soil analysis before conducting research) were reported that the soil contains 1.5% organic matter, 6.5 for pH level, 21.99 me100 g^{-1} soil of Cation Exchange Capacity (CEC), and 0.12%, 3.16 mg kg^{-1}, 0.18 me 100 g^{-1} soil for nitrogen, phosphate, and potassium, respectively. Data on organic matter, nitrogen, phosphate, and potassium are categorized as low to very low, as Prasetyo showed in Probolinggo [29]. This condition of low soil fertility will be discussed further in "future research" at the end of this manuscript.

2.2. Materials

The research used planting materials for local

Table 1. Climate condition on research location.

Average	Year					
	2015	2016	2017	2018	2019	2020
Number of rainy days (d)	9.50	15.08	13.25	10.83	8.00	14.00
Number of precipitation (mm)	2.44	170.89	152.33	119.66	99.25	162.87
Humidity (%)	79.25	81.88	80.75	76.00	73.79	76.29
Temperature (0C)	28.19	27.96	27.96	28.12	28.37	28.68
Atmospheric pressure (mb)*	1 012.35	1 011.80	1 011.12	1 011.17	1 011.59	1 008.95

*1 mb = 0.001 bar = 100 Pa, Source [30].

maize (*Zea mays* L.) var. Guluk-Guluk, of which groundnut (*Arachis hypogaea* L.) seeds were obtained from the direct harvest of local farmers and stem cuttings of Adira cultivar cassava (*Manihot esculenta* Crantz). The fertilizer consisted of cattle manure, burned rice husk, phonska, urea, trisodium phosphate (TSP), and potassium chloride (KCl). PGPR used in the research was a consortium of *P. fluorescens* and *B. polymyxa* from the Laboratory of Plant Disease and Pest Management of Pamekasan, Madura, East Java, Indonesia.

2.3. Experimental Design

Randomized Complete Block Design (RCBD) was used to arrange treatment for each experiment unit. This research consisted of four treatments, namely i) without PGPR application, ii) one-time application of PGPR at the planting time, iii) twice application of PGPR at the planting time, and 15 Days After Planting (DAP), iv) three times PGPR application at the planting time, 15 DAP, and 30 DAP. Each treatment had four replications. The placement of treatment in each group was done randomly.

2.4. Overview of the Experiment

Before planting, land preparation is done by clearing the land of weeds. Furthermore, 16 experimental plots were created, divided into four groups. The plot size was 8.30 m × 8.30 m. A 30 cm wide water channel with a depth of 30 cm was made between the plots. Each plot carried out minimum tillage, only tilling the soil in the row where corn, groundnut, and cassava will be planted. Basic fertilization used Bokhasi cattle manure fertilizer at 2 t ha^{-1}, burned rice husks 500 kg ha^{-1}, Phonska 200 kg ha^{-1}. Follow-up fertilization was carried out 20 days after planting using urea 150 kg ha^{-1}, and TSP 100 kg ha^{-1}. Planting in the experimental plot was arranged in three rows of groundnut, three rows of corn, three rows of groundnut, one row of cassava, three rows of groundnut, three rows of corn, and three rows of groundnut. The spacing of groundnut was 20 cm × 20 cm, corn 60 cm × 20 cm, and cassava with a distance of 90 cm in rows. Planting was carried out in the rainy season in 2019. The PGPR solution sprayed onto the soil was made by mixing 10 mL of PGPR in 1 L of water. Each plant was sprayed with 10 mL of PGPR solution. Each plot's need for a PGPR solution was calculated based on the total plant population. As the population of all plants per plot was 729, the need for PGPR solution was 7 290 mL. This solution was given evenly on the experimental plots. The time of PGPR application was adjusted to the treatment. In terms of determining changes in soil quality, soil chemical properties were observed, such as soil C-organic content, total N nutrient content, available P, exchangeable K, and cation exchange capacity. In addition, soil enzyme activities, namely nitrogenase and phosphatase enzymes, were observed. Crop production variables, namely corn, groundnut, and cassava, were observed per plot (kg ha^{-1}). The plant buds as forage for animal feed were also observed in fresh form (kg ha^{-1}).

2.5. Procedures of Soil Chemical Analysis

Soil samples were air-dried and ground for the preparation of chemical analysis. Nitrogen content was determined by the micro-Kjeldahl digestion method, available P was analyzed by the Bray-1 method, and K was extracted from soil using NH_4OAC 1N pH 7 and measured by a flame photometer. The wet combustion method was used to determine soil organic carbon, and cation exchange capacity was determined using the extractor NH_4OAC 1N pH 7 method. Soil nitrogenase activity was analyzed by Acetylene Reduction Assay (ARA) [31] and phosphatase activity was determined according to Zechmeister-Boltenstern [32].

2.6. Statistical Analysis

The obtained data were analyzed by analysis of variance (ANOVA). If the treatment effect is significant, the analysis is continued with multiple comparison analyses of the Least Significant Difference (LSD) with an error of 5%. Correlation between variables was analyzed using Pearson Product Moment correlation analysis, and all statistical analysis using SPSS-25 software [33, 34].

3. RESULTS AND DISCUSSION

The effects of PGPR application frequency on the intercropping system (groundnut-corn-cassava) showed an improvement in the crops' soil chemical properties, yield, and by-products. The detailed results are provided as follows.

3.1. Soil Chemical Properties

The PGPR application frequency significantly affected available phosphate levels and exchangeable potassium levels compared to those without PGPR treatment. However, there was no significant effect among the frequency of PGPR 1, 2, and 3 times of applications. The application of PGPR in dryland effectively increased the availability of these two nutrients, and the increase reached 96.97% to 129.11% and 108.57% to 128.57% for available phosphate and exchangeable potassium, respectively. This result shows the role of phosphate-solubilizing bacteria *P. flourescens* and *B. polymyxa* in the PGPR solution. *B. polymyxa* is a bacterium that can dissolve phosphate and is effective in helping to overcome water stress [35]. This statement is reinforced by the results of [36], which showed that *Bacillus* sp. could dissolve phosphate fertilizers. *P. fluorescens* inoculation also increased soil P availability and phosphatase activity and positively affected soil improvement [37]. Phosphate solubilizing bacteria could dissolve unavailable phosphate into available by producing phosphatase enzymes and organic acids [38]. In addition, phosphate dissolution can occur through the production of inorganic acids, a decrease in pH with the release of protons, and the production of exopolysaccharides [39].

The application of PGPR to the treatment two times and three times showed no significant effect when compared to PGPR treatment one time. PGPR in the one-time treatment has succeeded in changing phosphate to be available to plants, so the application of PGPR in treatment two times and three times is no longer practical. The reason is because the microbes also use the available phosphate to reproduce themselves. Thus, the enzyme activity increased significantly (Table 2), but the number of P available did not experience a significant increase [40]. The research conducted by [41] also showed similar results to this study. The reason behind this finding is the fact that microorganisms also need P to carry out their breeding. The improvement in exchangeable potassium found in this experiment is in line with the previous research conducted by [42] where several groups of bacteria (*Bacillus* and *Pseudomonas*) were reported to dissolve potassium. Insoluble potassium is converted into potassium available to plants which in general is through the mechanism of producing organic acids [43]. Thus, the increase of P and K in this study indicates that PGPR can be an environmental-friendly solution to overcome the limited availability of nutrients in dry land. This rhizosphere engineering strategy has become an important way to achieve sustainable crop production.

The application of PGPR frequency had no significant effect on N levels. However, *B. polymyxa* was reported to be able to fix N in the air in addition to dissolving phosphate [44]. Likewise, C-organic content and soil CEC were not significantly affected by the frequency of PGPR application. However, there is a tendency for these three variables to increase. PGPR microbial growth is influenced by organic compounds produced by plant roots [45, 46]. Increasing microbial biomass will increase soil organic matter because the main constituents of microbial bodies are protein, homo, and heteropolysaccharides [47]. In addition, microbes also produce polysaccharide compounds that add soil organic carbon and soil aggregation [48]. The application of PGPR improved the chemical properties of the dry land soil used in this study (Table 2). This result will support the sustainability of agricultural cultivation in dry land, as stated by Ojuederie *et al.* [13].

3.2. Enzyme Activity

The observed soil enzymes were phosphatase and nitrogenase. These enzymes were related to

Table 2. Soil chemical properties affected by PGPR application frequency on intercropping system.

PGPR Application	N total (%)	Available P (mg kg^{-1})		Exchangeable K (cmol kg^{-1})		C-Organic (%)	CEC (cmol kg^{-1})
Without PGPR	0.21	58.28	a	0.70	a	1.56	32.03
1 × PGPR	0.20	110.70	b	1.46	b	1.73	40.57
2 × PGPR	0.22	98.37	b	1.60	b	1.61	41.02
3 × PGPR	0.24	114.42	b	1.29	b	1.68	37.14

Note: Numbers with different letters in the same column are significantly different at the 95% probability level.

the PGPR given to the soil. The average activity of the two types of enzymes differed between treatments with the frequency of PGPR application. The more frequently given PGPR, the activity of the two enzymes increased (Table 3). The observed relationship between soil phosphatase enzyme activity and available phosphate showed a significant positive correlation with an r of 0.87. This result indicates the availability of nutrients for plants. Costa *et al.* [49] also reported that enzyme acid phosphatase was positively and significantly correlated with PGPR application. The phosphatase enzymes in soil are primarily derived from bacteria, fungi, and plants [50]. Its activity is influenced by temperature, soil pH [51, 52], soil organic carbon, and soil water content [53]. However, this study showed a weak correlation between phosphatase activity and C-organic levels. The correlation coefficient (r) was only 0.346. However, the phosphatase activity capable of providing relatively high available P (Table 2). The highest activity of nitrogenase enzymes was only 0.65 µg g^{-1} soil and showed a weak correlation with the total N content of the soil. The total N content of the soil in this study was in the low category [29]. The activity of microorganisms in the soil, including releasing enzymes and dissolving minerals such as phosphorus [54], also produces enzymes to degrade organic matter to produce nutrients available to plants [55]. The presence of these beneficial microorganisms helps the plant to produce biomass. In addition, Almeida *et al*. [56] had proven that soil enzyme activity could be used to indicate quality changes in degraded soils.

3.3. Crop Yield

Applying PGPR in groundnut-maize-cassava intercropping can increase groundnut production compared to treatment without PGPR (Table 4). The increase in production reached 30.08%. The twice application of PGPR showed the highest harvest dry production for all intercropped plants. Compared with no PGPR or control, dry-shelled corn production increased with PGPR application. The twice application of PGPR, namely at the time of planting and 15 DAP, corn production increased by 41.62%, and cassava production increased by 28.12%. This result indicates that rhizobacteria in the roots encouraged better plant growth so that crop production increased. Its role is to dissolve phosphate as well as increase exchangeable potassium (Table 2). This finding is in line with recent research which stated that the application of PGPR was able to enhance results and nutrition content on some crops such as horticulture and oil palm (*Elaeis guineensis* Jacq) [16, 57–59]. In addition, bacteria produce growth hormones such as indole acetic acid (IAA) that can stimulate growth and increase the yield of crops [24, 60, 61].

Table 3. The average of soil enzyme activity affected by PGPR application frequency on intercropping system.

PGPR Application	Phosphatase enzyme (µg g^{-1})		Nitrogenase enzyme (µg g^{-1})	
Without PGPR	0.47	a	0.38	a
1 × PGPR	0.63	b	0.45	b
2 × PGPR	0.78	c	0.58	b
3 × PGPR	0.88	c	0.65	b

Note: Numbers with different letters in the same column are significantly different at the 95% probability.

Table 4. Production of groundnut, corn, and cassava affected by PGPR application frequency on intercropping system.

PGPR Application	Groundnut (kg ha^{-1})		Maize (kg ha^{-1})		Cassava (kg ha^{-1})	
Without PGPR	323.40	a	1 255.96	a	224.25	a
1x PGPR	401.15	b	1 718.78	b	220.75	a
2x PGPR	428.87	b	1 778.28	b	287.32	b
3x PGPR	381.42	b	1 769.52	b	241.77	b

Note: Numbers with different letters in the same column are significantly different at the 95%.

3.4. Production of the Shoot as Animal Forage

The by-product of crops was utilized as livestock's forage to suffice the needs of feed. In Table 5, the statistical analysis result shows that the PGPR application did not show any significant difference which means it did not lead to an increase in shoot production. The observed forage production was a by-product of groundnut, maize, and cassava shoot. However, there was a tendency for growth in shoots compared to control or without PGPR. The increase can reach 20.38 %. Actually – with data that showed low soil fertility before the study – there is a conflict of interest between using agricultural waste in the form of shoots for animal feed and composting as a material to improve soil quality. Hence, it is interesting to study the benefits of these two things in mixed farming.

Some of the harvest waste should be incorporated into the soil to maintain the survival of PGPR and increase its population on dry land. Not all harvest waste is used for animal feed. In addition, livestock manure needs to be returned to the soil to increase organic matter input. Thus, it is expected that the soil organic matter content will increase and can be used as an energy source for the growth, maintenance of microorganism cells and the production of extracellular enzymes [62]. Components of cellulose and lignocellulosic organic matter are broken down into simple carbohydrates such as glucose for growth. Increased glucose levels cause increased bacterial growth [63]. Therefore, in the future, it is still necessary to research the application of PGPR combined with the return of organic matter from crop waste and livestock manure to improve soil quality so that plant performance is better and healthier and productivity enhancement. This recommendation is supported by research results [64–70] which reported that organic matter significantly improves soil quality. Goenadi *et al*. [68] explained on oil palm (*E. guineensis*), Sumatran *et al*. [69] reported the positive effect of the midrib decomposition of salak [*Salacca zalacca* (Gaertn.) Voss], while Pramulya *et al*. [70] wrote the addition of organic matter from the leaves of the shade plant of the lamtoro species [*Leucaena leucocephala* (Lam.) De Wit] in coffee plantations.

However, in mixed farming, the cost of transporting livestock manure from pens to farmland is a major consideration. It should be studied, for example, only 50 % of the remaining harvest is transported to the cattle shed as feed. The remaining 50 % was decomposed at the edge of agricultural land by adding local decomposing microbia [71, 72].

Biogas digesters (individual or communal) build near the cattle sheds [73, 74]. Daily, the digester is filled with manure and urine from cattle, which act as anaerobic fermentation. Likewise, all household organic waste (leftovers, kitchen wastes, tree leaves in the yard) is put into the digester with specific rules [75, 76]. This biogas digester is also connected to the latrines or septic tanks in people's homes [73, 77, 78] with this action, there are several advantages, namely renewable energy— clean energy for household kitchens and solid and liquid organic fertilizers [73,79, 80], which are helpful for plant productivity and soil amendments [81, 82] The amount of by-product as fertilizer from this digester is smaller, so it reduces transportation costs, but the quality is excellent [83, 84].

The results of other studies showed that the use of the PGPR bacterial consortium, which can provide N, P, and K was proven to increase the growth and physiological parameters of cereal plants. Several studies related to PGPR have also reported that Mycorrhiza has a positive effect on various plants and improves soil properties [85–88]. Therefore, besides the bacteria applied in

Table 5. The production of the shoot as animal forage after PGPR application per hectare.

Treatment	Groundnut (kg ha^{-1})	Maize (kg ha^{-1})	Cassava (kg ha^{-1})	Total (kg ha^{-1})
Without PGPR	323.40	1 225.96	224.25	1 682.61
1 × PGPR	401.15	1 718.78	220.75	2 340.68
2 × PGPR	428.87	1 778.28	287.32	2 494.47
3 × PGPR	381.42	1 769.52	241.77	2 392.71

Note: Numbers with different letters in the same column are significantly different at the 95% probability level.

this study, it is better to add other PGPR bacteria, namely N-fixing bacteria and mycorrhiza fungi, so that they become a consortium that is expected to increase the availability of nutrients and increase plant growth and production.

4. CONCLUSIONS

Applying PGPR significantly improved soil chemical properties and production in an intercropping system on dry land. The output of the shoot as forage for animal feed increased by 20.38 %. Enzyme activity increased when compared with no application of PGPR. The twice application of PGPR showed the best effect on soil quality, plant production, and fresh shoots. Using PGPR in intercropping plant cultivation in dry land is an alternative to maintaining soil quality. Thus, the sustainability of production can support the provision of food in the future.

5. ACKNOWLEDGEMENTS

The authors would like to thank to the Directorate of Research and Community Service under The Ministry of Research, Technology and Higher Education of Indonesia for funding through Applied Research Program (07/SP2H/PEN/MONO/ LPPM/UNIJA/2019).

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 171-179 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)1023

Pakistan Academy of Sciences

Research Article

Role of Hydrogen Peroxide (H_2O_2) and *Trichoderma koningii* in Reducing Root Rot Disease of Tomato Caused by *Fusarium solani*

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Abstract: Two isolates of the pathogenic fungus *Fusarium solani* the causative of tomato root rot disease named Fs1 and Fs2 were isolated from tomato plants infected with root rot disease. Results showed that both examined isolates had significant effects on the percentage of seed germination of tomato and damping-off disease. Thus, isolate Fs1 was more effective than isolate Fs2, as it recorded a percentage of germination and damping-off 56.6 and 71.6% respectively, compared to the control treatment, which recorded 100 and 0% respectively. It was found that the use of hydrogen peroxide (H_2O_2) at concentrations of 50, 100 and 200 ppm had a significant effect on the fungal radial growth, dry weight (DW) and sporulation of *F. solani*. Antifungal activity of H_2O_2 appeared even at the lowest concentration (50 ppm), which inhibited radial growth to 49.33%, and decreased the dry weight to 498 mg, and the sporulation to 3.75×10^6 spores. Additionally, the results indicated significant inhibitory effects of hydrogen peroxide (H_2O_2) and the bioagent *Trichoderma koningii* on *F. solani* growth. It was noticed that H_2O_2 has compatible effects with *T. koningii*, where the antagonistic ability of *T. koningii* against *F. solani* increased when the concentration of H_2O_2 had increased. The results revealed that the treatment *F. solani* + H_2O_2 (200 ppm) + *T. koningii* significantly reduced the percentage of damping-off and plant survival to 9.40 and 5.39% respectively, in comparison with the control treatment and *F. solani* alone treatment, which reached 20.22, 14.48, 34.32 and 67.41% respectively.

Keywords: Biological Control, Damping-off, *Fusarium solani*, Hydrogen Peroxide, Stimulation of Resistance, *Solanum lycopersicum L.*

1. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the most important economic vegetable produced in the world in terms of cultivated land area and consumption, with high nutrients including vitamins such as A, B and C, in addition to fructose, fats, protein, sucrose and some minerals including calcium, magnesium and phosphorus [1]. Tomato also contains antioxidant compounds including phenolic compounds, carotenoids and ascorbic acid [2].The total production of fresh tomato in the world reached to 180 million tons in 2019 with an area of 5 million hectares of agricultural land [3].The production of tomato crops in Iraq was estimated at 400,542 tons in an area of 79,269 dunums for the year 2020, and the production of Misan Governorate was estimated at 159,306 kg in an area of 53 dunums [4]. Tomato is a vulnerable plant that usaually infects with several pathogens, most importantly the pathogenic fungus *F. solani*, which causes wilting, root rot and damping-off diseases and causes great economic losses. Soil-borne diseases caused by many fungi such as *F. solani* were considered a problem for crop production, especially for vegetables. These pathogens often live for long periods on the host plant residues or organic matter in the soil. As these soil-borne fungi have become resistant to chemical fungicides, so, biological control is considered a safe and effective new alternative to fungicides in controlling plant diseases [5, 6].

Using the bioagent microorganisms for controlling soil borne fungi is considered as one of the most acceptable procedures, because of their safety for humans, animals, non-target organisms

Received: October 2023; Revised: May 2024; Accepted: June 2024
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and environment, while chemical fungicides may lead to contaminate the soil and have negative effects on human and animal health [7]. Several fungi have been applied for controlling plant diseases biologically. The important one is *Trichoderma* spp., this importance could be attributed to their inhibition mechanisms against pathogens, including parasitism, competition, producing antibiotics, etc. [8]. Many studies showed that *Trichoderma* promotes plant growth and development and has the ability to induce plant defense responses against different pathogens [9]. Zaghloul *et al.* [10] indicate that *Trichoderma* reduced root rot disease of tomato and stimulated a high plant production.

There are many previous studies were carried out in southern of Iraq on the ability of several species of the bioagent *Trichoderma* including *T. harzianum*, *T. longibrachiatum* for controlling damping off and root rot disease of tomato, eggplant and cucumber crops causing by *Fusarium solani* and *F. oxysporum*, [11-13]. Additionally, it was noticed that *T. harzianum, T. longibrachiatum*, *T. koningii* and *T. viride* significantly decreased the fusarium wilt disease of tomato which is caused by *F. oxysporum* f.sp. *lycopersici* [14, 15]. The use of inducing factors like hydrogen peroxide (H_2O_2), is one of the most important and modern methods for controlling plant diseases by activating different protective mechanisms designed to prevent the reproduction and spread of pathogens, such as defense mechanisms, rapid production of reactive oxygen types, modifications in cell wall composition and accumulation of secondary antimicrobial metabolites such as phytoalexins, they activate or synthesize defense peptides and proteins [16, 17]. The current study aimed to evaluate efficiency of the bioagent *T. koningii* and the inducing factor hydrogen peroxide (H_2O_2), individually or in combination for inhibiting the growth of the pathogenic fungus *F. solani*, as well as their ability to control root rot disease in tomato.

2. MATERIALS AND METHODS

The laboratorial experiments were performed in the laboratories of Plant Protection Department / College of Agriculture/University of Misan. The pot experiments were performed in the Agriculture College wooden canopy/University of Misan, in the year 2022.

2.1. Isolation and Identification of the Pathogenic Fungus *Fusarium solani*

Symptomatic plant materials were collected randomly from different fields. Infected roots were separated and washed carefully with running tap water and dried by using filter papers. Pieces with 1 cm length for each one was prepared and sterilized with 2% sodium hypochlorite (NaOCl) solution for 2-3 minutes, washed with sterile distilled water and dried again by using filter papers. Five sterilized pieces were put in each Petri dish containing sterilized PDA medium (200 gm potato, 20 gm dextrose and 20 gm Agar) with Chloramphenicol at a concentration of 250 mg/l. The plates were put in the incubator at a temperature of 25 ± 2 °C for a period of 3-5 days. The fungus was identified depending on its macro and micro scopic features according to Leslie and Summerell [18].

2.2. Pathogenicity Test

The pathogenicity of two isolates of *F. solani* was tested. Soil sterilized with formalin, left for about ten days and put in 1 kg plastic pots with equal quantities. The inoculum of *F. solani* which is grew on the seeds of local millet (*Panicum miliaceum* L.) was mixed well with the soil and added to each pot at a rate of 1% w/w. Millet seeds free from pathogenic fungus were added as control treatment at the same rate [19]. The soil was irrigated with water and the pots were covered with polyethylene bags for three days. Ten superficially sterilized seeds of tomato were put in each pot. Three replicates of each treatment including the control were applied. After one week, the germinated seed percentage was estimated as follows:

$$\%\ Germination = \frac{No.\ of\ geminated\ seeds}{Total\ No.\ of\ seeds} \times 100$$

The percentage of damping-off was calculated after one month as follows:

$$\%\ Damping - off = \frac{No.of\ dead\ seedlings}{Total\ No.of\ seedlings} \times 100$$

2.3. Effect of Hydrogen Peroxide (H_2O_2) on the Growth of *Fusarium solani*

This test was carried out using the most virulence isolates of the fungus *F. solani*. The technique of poisoned culture medium was used, where PDA medium was treated with concentrations of 50, 100 and 200 ppm of H_2O_2 and poured into Petri

dishes (9 cm diameter). A disc (0.5 cm diameter) was taken from an edge of 7-day-old pure colony of *F. solani* and put in the center of each plate, the control treatment was left without adding H_2O_2. Each treatment was replicated for three times. All plates were kept in incubator at a temperature of 25 ± 2 °C. The fungal radial growth was measured when the fungal growth in the control treatment reached the edge of the plate, the percentage of fungal growth was calculated by using the equation suggested by Ahmed [20] as follows:

$$Reduction\ in\ linear\ growth = \frac{G1 - G2}{G1} \times 100$$

Where, G1 = Linear growth of the fungus in the control. G2 = Linear growth of the fungus in the treatment.

2.4. Effect of Hydrogen Peroxide (H_2O_2) on Dry Weight of *Fusarium solani* Biomass

The toxicity of hydrogen peroxide (H_2O_2) at concentrations of 50, 100 and 200 ppm were examined against the growth of *F. solani* in potato dextrose broth (PDB) medium (three flasks for each concentration). Equal discs with a diameter of 0.5 cm for each one were taken from an edge of 7-day-old colony of *F. solani* and added to 250 ml flasks containing 50 ml sterile PDB with concentrations of 50, 100 and 200 ppm H_2O_2. The control treatment had no H_2O_2. All flasks were transferred to the incubator and incubated for ten days at a temperature of 25 ± 2 °C [21]. The mass dry weight (mg MDW per 100 ml liquid medium) of different treatments was measured after separating the fungal mass by filtration of the PDB with filter paper. The samples were dried at 60 °C for two days by using the oven. The percentage of fungal growth inhibition was calculated according to Sutton *et al*. [22] as follows:

$$\%Inhibition = \frac{MDW of\ control - MDW of\ treatment}{MDW of\ control} \times 100$$

2.5. Effect of Hydrogen Peroxide (H_2O_2) on Sporulation Rate of *Fusarium solani*

After calculating the growth rate of the different treatments in the media, *F. solani* isolate was grown in 9 cm diameter Petri dishes supplied with PDA medium treated with three concentrations of hydrogen peroxide, 50, 100 and 200 PPM, with three replicates for each concentration, and incubated for 10 days at a temperature of 25 ± 2 °C. One disc with a diameter of 0.5 cm was taken by a sterile cork borer and put in each test tube containing 10 ml of sterile distilled water. Each treatment was replicated three times. All tubes were shaken well for about five minutes with a vibrator to release spores from the conidiophores. The rate of sporulation of *F. solani* in 1 ml was calculated by using a hemocytometer, and the rate of the number of spores in a 0.5 cm disc was calculated by multiplying the rate of the number of spores in 1 ml × 10.

2.6. *In vitro* Effect of Combination of H_2O_2 and *Trichoderma koningii* on the Growth of *Fusarium solani*

Hydrogen peroxide (H_2O_2) at the concentrations of 50, 100 and 200 PPM were added to sterile PDA medium and poured into Petri dishes (20 ml /dish) and left to solidify. Each plate was divided into two equal parts. A disc with a diameter of 0.5 cm was taken from the edge of 7-day old colony of the bioagent *T. koningii* and placed in the center of one part of the plate, while the other part has been inoculated with a 0.5 cm disc of the pathogenic fungus *F. solani*. A control treatment was applied by inoculating the center of the Petri dish with a 0.5 cm disc of *F. solani* only. Each treatment was triplicated. All plates were transferred to the incubator with a degree of 25 ± 2 °C. Both pathogenic and bioagent fungal growth was measured regularly. When the growth of *F. solani* in control treatment reached the plate edge, the percentage of fungal growth inhibition (MGI%) for all treatments was estimated according to EL-Ashmony *et al*. [21] according to the following equation:

$$MGI\% = \frac{(dc - dt)}{dc} \times 100$$

Where, dc = fungal colony diameter in the control, dt= fungal colony diameter in the treatment.

2.7. Preparation of the Fungal Inoculum of the Pathogenic and the Bioagent Fungi

Inoculum of *F. solani* and the bioagent *T. koningii* were prepared according to [19]. as follows: Local millet seeds (*Panicum miliaceum* L.) were washed with tap water carefully. 150 gm of the seeds were put in each 250 ml flask and autoclaved for 1h at a temperature of 121 °C and a pressure of

15 pound/inch^2. Each flask was inoculated with 5 discs taken from the edge of a 7-day-old colony of each of *F. solani* and *T. koningii* separately. Finally, the flasks were transferred to the incubator and incubated for 14 days at a temperature of 25 ± 2 °C with shaking them well every two days to distribute the fungal inoculum on the seed surface.

2.8. The Efficacy of Hydrogen Peroxide (H_2O_2) and Bioagent *T. koningii* Treatments in Reducing *F. solani* the Causal Agent of Tomato Root Rot Disease in Pots

Plastic pots (5 kg capacity) containing sterilized soil with formalin were prepared. The bioagent *T. koningii* grown on millet seeds was added to the soil with an average of 1% w/w, mixed well and left for three days with irrigation [19]. The pathogenic fungus *F. solani* was added to the soil with same rate after three days with continuous irrigation. After another three days, ten seeds of local variety of tomato treated with two concentrations of hydrogen peroxide (H_2O_2) 50 and 200 ppm each separately were planted in each pot. Control treatment was left without adding anything. Each treatment was triplicated. All pots had been distributed in the plastic house randomly. The pots were irrigated as the field capacity. The treatments were conducted as follows:

Control, pathogenic fungus *F. solani*, bioagent *T. koningii*, H_2O_2 (50 ppm), H_2O_2 (200 ppm), *F. solani* + *T. koningii, F. solani* + H_2O_2 (50 ppm), *F. solani* + H_2O_2 (200 ppm), *F. solani* + *T. koningii* + H_2O_2 (50 ppm), *F. solani* + *T. koningii* + H_2O_2 (200 ppm).

After 30 days, the percentage of damping-off was calculated, while the percentage of disease severity was calculated after 90 days based on five-grade disease index suggested by Dorrance *et al*. [23], where 0 = no root rot appearance, 1 = appearance ulceration or visible discoloration on 1-33% of the roots, 2 = root rot in approximately 34-50%, 3 = root rot in approximately 51-80%, 4 = root rot more than 81% or plant death.

The disease severity index (DSI) was estimated according to Liu *et al*. [24] as the following equation:

$$DSI = \frac{\Sigma d}{d\,max \times n} \times 100$$

Where, d represents the disease rating possible, d max refers to the maximum disease rating and n represents the total number of plants examined in each replicate.

Statistical analysis

A completely randomized design (CRD) was applied for laboratory and pot experiments. To compare the means, the least significant difference (LSD) test was applied with a probability 0.01 for laboratory experiments and 0.05 for pot experiments. Statistical program (SPSS) version 23 was used for analyzing data.

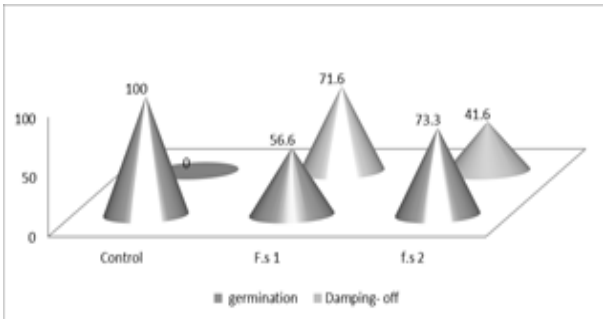
3. RESULTS AND DISCUSSION

3.1. Isolation and Identification of *Fusarium solani*

Two isolates of *F. solani* were isolated from the infected roots of tomato. The colony of *F. solani* showed a white to cream mycelium on PDA, reverse pale, septate hyphae, macroconidia straight with 3-4 septa, microconidia fusiform or kidney shaped with no or one septum, Chlamydospores spherical or ovoid shaped, produced singly or in pairs. These characteristics have been agreed with [25, 26].

3.2. Pathogenicity Test of the Two Isolates of *F. solani* in Pots

The results (Figure 1) elucidated that the two isolates of *F. solani* (Fs1 and Fs2) had a significant effect on tomato seeds germination percentage and damping-off percentage. It was found that the percent of germination and damping-off in the treatment Fs1 were 56.6 and 71.6% respectively, and significantly differed from the isolate Fs2 which recorded 73.3 and 41.6% respectively. Both isolates were significantly differed from the control treatment, which amounted to 100 and 0% respectively. This



L.S.D $_{0.01}$ (Germination) = 6.8 L.S.D $_{0.01}$ (Damping-off) = 16.1

Fig. 1. Pathogenicity test of *F. solani* isolates in the pots.

result agreed with [12, 13], who confirmed that *F. solani* caused root rot disease on cucumber and eggplant in the pots. The differences between the effect of both isolates could be attributed to the genetic differences between them and differences in their ability to produce fusaric acid, the potent phytotoxin [27], as well as its production of pectin and cellulose-degrading enzymes such as phosphatase, pectinase, cellulase, methylesterase and pectinmethylhydrase and production of toxins, phenolic and glycosidic compounds [28].

3.3. *In vitro* Effect of Hydrogen Peroxide (H_2O_2) on the Growth of *F. solani*

Table (1) showed the effect of H_2O_2 on fungal radial growth, fungal dry weight (DW) and sporulation of *F. solani* which varied according to the examined concentrations, the concentration 50 ppm had the lowest effect where the fungal radial growth, fungal dry weight and sporulation amounted to 49.33 mm, 498 mg and 3.75×10^6 spores/ml respectively, while the concentration 200 ppm gave a highest effect and significantly reduced the fungal radial growth, fungal dry weight and sporulation, as they reached 22.04 mm, 124 mg and 0.93×10^6 spores/ml respectively, and significantly differed from other concentration and control treatments that recorded 85.30 mm, 738.00 mg, and 6.98×10^6 spores/ml, respectively. It was also noticed that the inhibition percentage of *F. solani* had significantly increased with the increasing of H_2O_2 concentrations, thus, our results are in accordance with the results of El-Ashmony *et al*. [21] who found that H_2O_2 had affected on the pathogen growth significantly. The effect of H_2O_2 may be explained by their reactivity, which had been toxic to cells at high concentrations [29]. Finnegan *et al*. [30] explained that H_2O_2 also may contains multiple models of structural oxidation and stages different oxidative stresses of proteins, amino acids and differences in its effect towards the different enzymes produced by microorganisms.

3.4. Effect of H_2O_2 and *Trichoderma koningii* Treatment on *F. solani* Growth *In vitro*

Table (2) showed that the treatment of *T. koningii* increased the inhibition percentage of *F. solani* when it was interacted with H_2O_2. It was found that increasing of H_2O_2 concentration led to increase the inhibitory effect of the bioagent *T. koningii* against *F. solani*, where the concentration 200 ppm gave the highest percentage which reached 58.48% compared to the control treatment, which recorded 0.00. The high antagonistic ability of *T. koningii*

Table 1. *In vitro* effect of H_2O_2 on fungal growth, fungal dry weight and sporulation of *F. solani.*

Treatments	Fungal radial growth (mm)	% Inhibition	Fungal dry weight (mg/50ml) liquid medium	Sporulation (X10^6 spores/ml)
Control	85.30	0.00	738	6.98
H_2O_2 (50 ppm)	49.33	45.03	498	3.75
H_2O_2 (100 ppm)	37.24	61.47	278	2.37
H_2O_2 (200 ppm)	22.04	78.20	124	0.93
$L.S.D_{(0.01)}$	2.074	2.040	139.6	1.228

Table 2. Effect of interaction of H_2O_2 and *Trichoderma koningii* on the growth of *F. solani in vitro.*

Treatments	% Inhibition of linear growth of *F. solani*
Control	0.00
H_2O_2 (50 ppm) + *T. koningii*	42.22
H_2O_2 (100 ppm) + *T. koningii*	52.32
H_2O_2 (200 ppm) + *T. koningii*	58.48
$L.S.D_{(0.01)}$	1.279

may be attributed to its production of several enzymes that can degrade cell wall and release a number of mycotoxins that can inhibit the growth of the pathogen [31]. Also, it was found from previous studies that the different species of *Trichoderma* can produce many extracellular enzymes including protease, cellulase, poly galacturonase, B-1,3-gluconase and chitinase, and produce many antibiotics like trichodermin, viridin, besides, they can produce toxic compounds like gliotoxin which can reduce pathogenic fungi growth [32,33].

3.5. Effect of Hydrogen Peroxide (H_2O_2) and Bioagent *T. koningii* Treatment in Reducing Tomato Damping-off Caused by *F. solani* and Survival Plants in Pots

It was noticed from Table (3) that there was a significant decrease in damping-off and survival plants depending on the response of *F. solani* to different concentrations of H_2O_2 alone or in combination with the bioagent *T. koningii*, as the highest concentration of H_2O_2 (200 ppm) recorded a significant decrease in damping-off and survival plant which reached17.78 and 10.55%, respectively. *T. koningii* also reduced damping-off and survival plants significantly to 17.48 and 10.70% in comparison with control and pathogen treatments which amounted to 20.22, 14.48, 34.32 and 67.41% respectively. Additionally, the combination treatment of *F. solani* + H_2O_2 (200 ppm) + *T. koningii* was found to be the best treatment which led to decrease the percentage of damping-off and plant survival significantly to 9.40 and 5.39% respectively. These results agreed with Al-Abbas and Salih [12], Al-Mansoury and Salih [13], and Hassan and Salih [14, 15], who confirmed that *Trichoderma* species such as *T. harzianum*, *T. longibrachiatum*, *T. koningii*, and *T. viride* led to decrease root rot, damping-off and fusarium wilt diseases in okra, cucumber, eggplant and tomato caused by *F. oxysporum* and *F. solani* significantly compared to the pathogen treatments. The results also agreed with Abdel-Monaim *et al*. [17] who mentioned that the high rates of tomato damping-off disease before and after emergence caused by *Pythium* sp., *F. oxysporum* and *F. solani* were reduced significantly as a response to inducing factor H_2O_2. Copes [34] indicated that H_2O_2 activated the plant defense mechanisms by increasing lignin and suberin contents in plant and increasing the plant defending against pathogens, H_2O_2 also, plays an essential role in strengthening cell walls at the site of the attack by pathogens. The reason for decreasing disease severity and root rot may be attributed to the presence of *T. koningii* which can protect the plant from the disease infection, promote its growth and supply some essential minerals [35]. It was found from some previous studies that *Trichoderma* spp. stimulate the plants

Table 3. Effect of interaction of H_2O_2 and bioagent *T. koningii* in reducing damping-off disease of tomato causing by *F. solani* and survival plants in pots under greenhouse conditions.

Treatments	% Damping-off	Survival plants
Control	20.22	14.48
F. solani	34.32	67.41
T. koningii	17.48	10.70
H_2O_2 (50 ppm)	19.22	12.80
H_2O_2 (200 ppm)	17.78	10.55
F. solani + *T. koningii*	25.33	46.22
F. solani+ H_2O_2 (50 ppm)	30.75	54.75
F. solani+ H_2O_2 (200 ppm)	15.15	14.44
F. solani + H_2O_2 (50 ppm) + *T. koningii*	12.36	7.75
F. solani + H_2O_2 (200 ppm) + *T. koningii*	9.40	5.39
$L.S.D._{(0.05)}$	1.49	1.59

to make changes in their metabolism and lead to changes in the plant response to pathogens and environmental stress and help the plants to tolerate the hard-environmental conditions, also they produce some compounds that have toxic action against the pathogens like viridin, koninginin, azaphilones, pyrones and steroids [36-38].

4. CONCLUSIONS

The main objective of this study was to find the best procedure to reduce the pathogen *F. solani* the causative of damping of and root rot on tomato plants by using the inducing agent hydrogen peroxide (H_2O_2) and the bioagent *T. koningii*. Results revealed that the disease severity had been decreased with the increasing of the hydrogen peroxide concentration, as well as using the bioagent *T. koningii*, which gave positive results in controlling and reducing this disease when it used alone or in combination with the inducing factor (H_2O_2) at different concentrations. Damping-off disease decreased significantly when the bioagent *T. koningii* and H_2O_2 were used together.

5. ACKNOWLEDGEMENT

We are very grateful to the Head of the Plant Protection Department for providing all the laboratory equipment and chemicals. We also thank Dr. Durgham Sabeeh Karim Al-Taie for statistically analyzing the data.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 181-188 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)789

Pakistan Academy of Sciences

Research Article

Medicinal Efficacy of *Tinospora cordifolia, Caesalpinia bonduc,* and *Quercus infectoria* Extracts

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Abstract: Medicinal plants have therapeutic value because of the chemical substances present in them. This study was performed to explore the antibacterial and antioxidant potential of three medicinally important plants, such as *Caesalpinia bonduc* (karanjuwa), *Tinospora cordifolia* (Giloy), and *Quercus infectoria* (Majuphal) extract; that are locally used for the cure of various diseases, particularly dengue fever. To study the anti-bacterial, phytochemical and antioxidative potential of these plants, plant extracts were made in ethanol, methanol, and ethyl acetate. Antibacterial assay revealed that all the selected plant extracts of *C. bonduc, T. cordifolia,* and *Q. infectoria* variably inhibited the growth of test bacterial strains. The maximum antibacterial activity observed for the ethyl acetate extract of *T. cordifolia* was against *Bacillus* (41 mm) and *Pseudomonas* (38 mm). Phytochemical analysis of extracts of *C. bonduc, T. cordifolia,* and *Q. infectoria* revealed the presence of alkaloids, quinones, flavonoids, phenols, and reducing sugars. Antioxidative potential of the extracts of *C. bonduc, T. cordifolia,* and *Q. infectoria* was revealed by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (66%, 62% and 77%), phosphomolybdate (480, 520 and 490 µg/ml), showed ferric reducing antioxidant power (FRAP) assay (266%, 371% and 265%), and total flavonoid content (75%, 170% and 155%) assay. Due to significant antibacterial and antioxidant potential, the extract of *T. cordifolia* was subjected to Thin-layer chromatography (TLC) analysis that confirmed the presence of different bioactive components in the extract of *T. cordifolia* (Giloy). Gas Chromatography-Mass spectrophotometry (GCMS) analysis of the bioactive fractions revealed Phthalic acid and Benzene acetic acid as the compounds responsible for imparting good antibacterial and antioxidative potential to the ethyl acetate extract of *T. cordifolia*. These findings highlight the potential of *T. cordifolia* as a valuable medicinal plant for the development of future antimicrobial drugs.

Keywords: *Tinospora cordifolia*, *Caesalpinia bonduc*, *Quercus infectoria,* Antibacterial Activity, Phytochemicals, Antioxidant Potential, Phthalic Acid, Benzene Acetic Acid.

1. INTRODUCTION

Human history reveals that the utilization of different plants for the curing of various diseases started thousands of years ago. Plants that can cure infectious diseases are called medicinal plants [1]. Local people in several countries, like China and Egypt, started to use herbal medicines for curing infectious and non-infectious diseases [2]. Various synthetic drugs from the bioactive compounds of these remedial plants were extracted that play a very important part in the development of modern medicines. This is the reason that the focus of research has been on folk medicine to treat infectious diseases [3]. It is crucial to comprehend the significance of the new medication and make an effort to isolate numerous bioactive compounds found in specific medicinal plants. In this study, three medicinal plants: *Caesalpinia bonduc, Quercus infectoria and Tinospora cordifolia* were used. *C. bonduc* plant is a member of the Caesalpiniaceae family [4]. Chemical analysis of this plant has highlighted its significance in the pharmacological field, owing to its diverse range of bioactive properties. Widely found in hot and humid climates, this therapeutic plant, also known as nicker or fever nut, has been extensively utilized in traditional medicine to cure numerous ailments [5]. *Q. infectoria* is a member of the family Fagaceae. It is a small-sized tree that is widely spread in Iran, Asia, and different areas of Greece. On this tree, galls arise on young branches when it is attacked

Received: November 2022; Revised: April 2023; Accepted: June 2024
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by the gall-wasp. In Malaysia, the galls are known as *manjakani* and *majuphal* locally. It also has great importance in Indian folk medicines, where it is extensively used in powder form for the cure of various tooth-related diseases [6, 7].

T. cordifolia has a common name, Gilroy belonging to the family Menispermaceae. It has a woody shrub that was native to India and is found in Sri Lanka and Burma in great numbers [8]. Male flowers are usually bunched, and the female flower is isolated in the racemes or racemose panicles. The flowering season expands over summers and winters [9]. *T. cordifolia* has different local names like Gilroy, Guduchi, etc. *T. cordifolia* has phytochemicals that belong to diverse classes of compounds, like phenols, flavonoids alkaloids, and terpenoids [10]. Numerous kinds of bioactive compounds have been extracted worldwide from different parts of *T. cordifolia* plant.

Dengue fever is a mosquito borne viral disease. Nature has a massive pool of bioactive materials that can be used directly as pharmaceuticals, or their derivatives can be utilized as effective agents to combat dengue. Presently, there are no specific treatments. But there are few medicinal herbs, such as *T. cordifolia,* which have presented some good pharmacological properties against dengue [11]. Hence, the present study was devised to investigate the phytochemicals, antibacterial, and antioxidative potential of extracts from three medicinal plants (*T. cordifolia* (wild), *C. bonduc*, and *Q. infectoria*) commonly used for dengue fever treatment. This exploration aims to lay the groundwork for their potential application in future studies focused on developing effective antiviral medications.

2. MATERIALS AND METHODS

2.1. Preparation of Plant Extracts

Seed, bark, and galls of three different plants, i.e., *C. bonduc, T. cordifolia* (wild), and *Q. infectoria,* were collected from the local market in Lahore, Pakistan. These plant samples were stored in Institute of Microbiology and Molecular Genetics (IMMG) under the voucher numbers MMG-IM-30, MMG-IM-31, MMG-IM32 and MMG-IM-33. Seed, bark, and galls were thoroughly washed by sterilized distilled water 3-4 times, air-dried under shade, and crushed in powdered form. The powered material (20 grams) of each plant was dipped in the respective solvent (100 ml) for 48 hours; ethyl acetate was used for *T. cordifolia,* and ethanol was used for *C. bonduc* and *Q. infectoria.* The extracts were filtered and finally dried with the help of a rotary evaporator to make them concentrated for further use.

2.2. Antibacterial Activity of Plant Extracts

The antibacterial potential of ethyl acetate and ethanol plant extracts can be measured by an agar well-diffusion assay [12]. For this purpose, 100 µl of 24 hours old culture (OD_{600nm} 0.5) of *Bacillus* (JQ013099) and *Pseudomonas* (KC881031) were spread on Muller Hinton agar (MH) plates to check the antibacterial activity of selected plant extracts. In the wells, 50 µl of the previously prepared plant extract (20%) was added. These plates were incubated at 37 °C for 24 hours and zones of inhibition (mm) were measured. Ethanol and ethyl acetate were used as negative controls, whereas ampicillin (10 µg/ml) was a positive control**.** The experiment was done in triplicate.

2.3. Phytochemical Analysis

Selected plant extracts were subjected to the following tests for the screening of various phytochemicals present in these extracts [13].

2.3.1. Alkaloids

Three to four drops of the Wagner reagent were added to one ml of plant extracts. The development of a reddish-brown color indicated the alkaloids.

2.3.2. Phenols

To 1 ml of plant extract, a few drops of ferric chloride solution (5%) were added as a reagent. The appearance of the deep blue color confirmed the phenols.

2.3.3. Flavonoids

Flavonoids are a group of polyphenols that are especially present in plants. A 20% sodium hydroxide solution (0.5 ml) was mixed with plant extract (1 ml). Yellow coloration showed a positive test that became colorless when diluted

hydrochloric acid was added.

2.3.4. Terpenoids

The addition of one ml of plant extract to two ml of chloroform, followed by the careful incorporation of three ml of concentrated H_2SO_4, resulted in the appearance of a red color, indicating the presence of terpenoids [14].

2.3.5. Reducing sugars

For this, 100 µl of the Benedict reagent was added to one ml of plant extract and then boiled for five minutes in a water bath. The appearance of red precipitates confirmed the occurrence of reducing sugars in plant extracts [15].

2.3.6. Steroids

One ml of chloroform was added to an equal volume of plant extract, and then one ml of conc. sulfuric acid was added at the end. A red color appeared, which indicated the steroids.

2.3.7. Tannins

For the detection of tannins, one ml of water is mixed with one ml of plant extract. Later on, a small amount of solution of $FeCl_3$ was added. Development of the green color showed the tannins.

2.4. Antioxidative Analysis of Plant Extracts

The presence of antioxidants in selected medicinal plants was confirmed by performing following four different assays:

1. Radical scavenging capacity by DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay
2. Phosphomolybdate assay
3. FRAP (Ferric Reducing Antioxidant Power) assay
4. Flavonoid content

2.4.1. Radical scavenging capacity by DPPH assay

Radical scavenging capacity was confirmed by following the method of Dilshad *et al.* [13]. Briefly, a standard solution of DPPH was prepared by the addition of DPPH (24 mg) in methanol (100 ml), and the optical density of the working solution was measured at 517 nm. After 60 min, the percentage radical scavenging capability was evaluated. Working solution (3 ml) was mixed with plant extract (100 µl), incubated in the dark, and optical density was measured.

2.4.2. Phosphomolybdate assay

The phosphomolybdate assay is commonly employed to evaluate the total antioxidant capacity of the extract. In this procedure, the plant extract (0.1 ml) was combined with a reagent solution containing 28 mM Na_3PO_4, 0.6 M sulfuric acid, and 4 mM $(NH_4)_6Mo_7O_{24}$. Subsequently, the test tubes were incubated in a water bath at 95 °C for one and a half hours. Following incubation, the test tubes were cooled, and the optical density was measured at 765 nm. Ascorbic acid was taken as the standard [15].

2.4.3. FRAP assay

Ferric reducing capacity of antioxidants present in plant extracts was confirmed by following the method of Jan *et al.* [15].

2.4.4. Flavonoid content

The estimation of flavonoid content was done by following the method of Dilshad *et al*. [16]. In this process, flavonoids form a complex with aluminum when reduced by antioxidants, resulting in color production. Rutin served as the standard in this assay, with concentrations ranging from 75 to 750 mg/L.

2.5. Thin Layer Chromatography (TLC)

In order to separate the different components of crude extract, TLC was employed by following the slightly modified method of Dilshad *et al.* [16]. A crude extract of *T. cordifolia* was spotted and developed in a solvent system (chloroform 8: methanol 1). Afterward, the TLC plate was taken out of the TLC tank and left to dry in the air. The separated components were then marked under both long and short UV rays. The spots observed were labeled from 1 to 7, and their *Rf* values were determined. The labeled spots were then scraped off from the TLC plate and placed in glass vials

containing ethyl acetate. After 24 hours, the extracts were filtered and the filtrate was allowed to evaporate until it dried.

2.6. Antibacterial Activity of TLC Spots

The antibacterial activity of these spots was checked against *Bacillus* (JQ013099) and *Pseudomonas* (KC881030). A disc diffusion assay was used to measure the antibacterial activity of the selected spots [13]. For this assay, ethyl acetate and ampicillin (10 µg/ml) were used as negative and positive controls, respectively.

2.7. Gas Chromatography Mass Spectrophotometry (GCMS) Analysis

GCMS analysis was performed to find the partially purified bioactive compound (spot numbers 2 and 5) that had antibacterial activity in the extract of *T. cordifolia*. The length of the column was 30 m, and helium was used as a carrier gas. The injection port temperature was maintained at 280 °C, and 2 µl of the sample was introduced at a flow rate of 1 ml/min. Subsequently, the temperature was increased to approximately 350 °C. The column was run for nearly 50 minutes leading to the detection of distinct peaks.

3. RESULTS

3.1. Selected Medicinal Plants

Medicinal plants, i.e., *C. bonduc (Karanjuwa), T. cordifolia (Giloy), and Q. infectoria (Majuphal),* which are being used locally for the treatment of flu and associated symptoms, particularly dengue fever, were procured from a local market in Lahore, Pakistan.

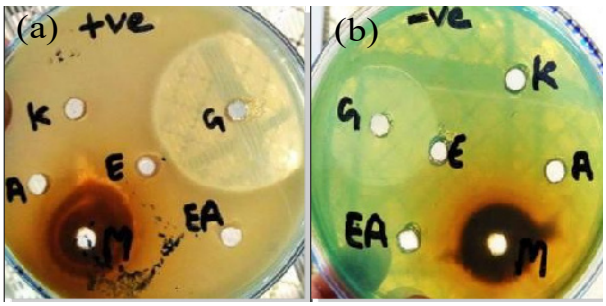


Fig. 1. Antibacterial activity of selected medicinal plant extracts: *T. cordifolia* (G); *Q. infectoria* (M) and *C. bonduc* (K) against (a) gram-positive and (b) gram-negative bacterial strain.

3.2. Antibacterial Activity of Plant Extracts

The maximum inhibition zone was shown by *T. cordifolia* (G) against both gram-positive (41 mm) and gram-negative (38 mm) strains. *Q. infectoria* (M) also inhibited the growth of both bacteria, as shown by their zones of inhibition against gram-positive (24 mm) and gram-negative (23 mm) bacteria (Figure 1). While, *C. bonduc* (K) demonstrated no inhibition potential (Table 1).

3.3. Phytochemical Analysis

Extracts of *T. cordifolia, C. bonduc, and Q. infectoria* were subjected to phytochemical analysis to find the different bioactive compounds present in them. All the plant extracts showed the presence of alkaloids, phenols, flavonoids, and sugars; whereas tannins, steroids, and pholobatanins were not found in *T. cordifolia* extract (Table 1).

3.4. Antioxidative Potential of Plant Extracts

The antioxidant potential of selected plant extracts was confirmed by different assays. Generally, the antioxidant ability of *T. cordifolia* was higher as compared to other selected plant extracts. The highest radical scavenging ability (DPPH) was detected in the extract of *Q. infectoria* (77%). While, the maximum amount of ascorbic acid (AA) (520 µg/ml) and highest ferric reduction potential (371%) were shown by the extract of *T. cordifolia*. The amount of total flavonoid concentration was also higher (170%) in the case of *T. cordifolia,* whereas *C. bonduc,* has shown the least (75%) concentration (Table 1). *T. cordifolia* exhibited significant antibacterial and antioxidative potential than *C. bonduc, and Q. infectoria;* therefore, it was selected for further analysis.

3.5. Thin-Layer Chromatography

Different bioactive components present in a crude plant extract can be separated with TLC. *T. cordifolia* had shown greater antibacterial and antioxidant potential than the rest of the tested extracts. The separated components were marked and treated with iodine. Seven spots were separated by means of TLC, as shown in Figure 2.

3.6. Antibacterial Activity of TLC Spots

Out of seven spots, two spot (2 and 5) gave the maximum antibacterial activity. Spot 2, gave a 10 mm inhibition zone with the *Rf* of 0.20, while spot 5, with *Rf* value of 0.55, showed 8 mm zone of inhibition. These two spots were further analyzed by gas chromatography mass spectrometry (Figure 3).

3.7. GC-MS Analysis

GC-MS analysis identified the compounds based on their mass. Analysis of these components presented two peaks at a retention time of 25.33 and 32.36 minutes. This analysis revealed that these components could be benzene acetic acid and phthalic acid, respectively (Table 2).

4. DISCUSSION

Throughout ancient history, plants have been utilized for their medicinal benefits across diverse cultures. The therapeutic properties of plants come from their bioactive constituents, which can be sourced from any part of the plant. Research has increasingly highlighted the potential of these medicinal plant extracts in uncovering innovative medicines, a need that continues to intensify with each passing day [17].

In the present study, *T. cordifolia, C. bonduc,* and *Q. infectoria* extracts were explored for their antibacterial and antioxidative potential due to the local use of these plants for the treatment of dengue fever. Phytochemical analysis of these extracts revealed the presence of different metabolites, such

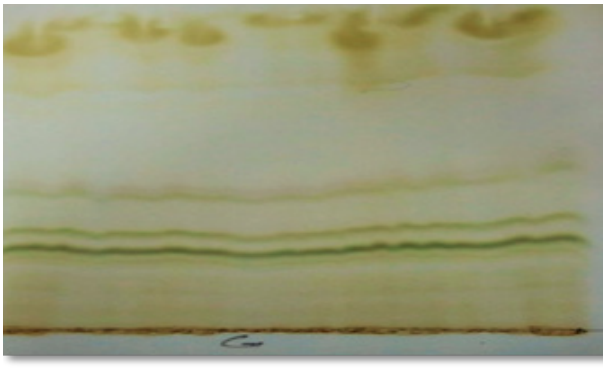


Fig. 2. TLC of the ethyl acetate extract of *T. cordifolia.*

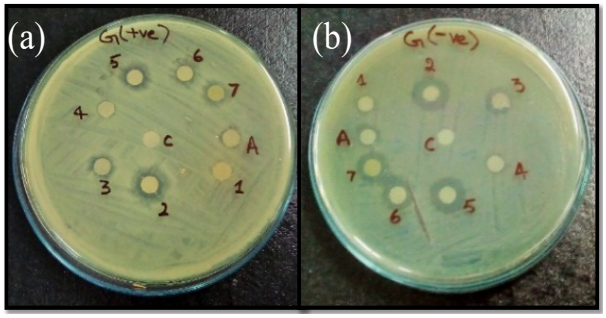


Fig. 3. Antibacterial activity of partially purified bioactive components of *T. cordifolia* extract against (a) gram positive (b) gram negative bacterial strain.

Table 1. Phytochemical analysis, antibacterial and antioxidant activity of selected medicinal plant extracts.

Extracts	Phytochemical Tests									Antibacterial Activity		Antioxidant Activity			
	Alkaloids	Phenols	Quinones	Terpenoids	Steroids	Phlobatanins	Sugars	Tannins	Flavonoids	Gram Positive (mm)	Gram Negative (mm)	DPPH (%)	Phosphomolybdate assay (µg/ml)	FRAP assay (%)	TFC (%)
T. cordifolia	+	+	+	-	-	-	+	-	+	41 ± 0.55	38 ± 0.32	62	520	371	170
C. bonduc	+	+	-	+	+	+	+	+	-	-	-	66	480	266	75
Q. infectoria	+	+	-	-	-	-	+	+	-	24 ± 0.17	23 ± 0.12	77	490	265	155

Mean of three replicates ± standard error of the mean.

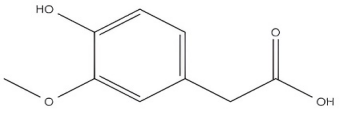
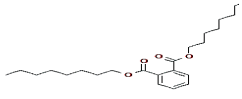
as saponins, alkaloids, amino acids, flavonoids, terpenoids, and tannins. The composition of different extracts was different because of the varied solvents in which extracts were made [18]. Though, ethanol is considered to be more polar as compared to ethyl acetate, which makes ethanol a better solvent to dissolve most of the bioactive compounds of plants in it as compared to ethyl acetate [19].

The antibacterial activity of extracts of *T. cordifolia* (Giloy), *C. bonduc* (Karenjuwa), and *Q. infectoria* (Majuphal) showed the presence of effective antibacterial compounds in them. Maximum growth inhibition for both strains (gram-positive and gram-negative) was observed by *T. cordifolia,* while minimum inhibition was shown by *C. bonduc*. The growth of gram-positive bacterial strain was inhibited slightly more than that of the gram-negative strain. The difference in inhibition potential for *Bacillus* and *Pseudomonas* is due to differences in the cell wall composition of both cell types. Gram-negative bacteria (*Pseudomonas*) have an extra layer outside of the cell wall, which makes them difficult to target by the different antibacterial compounds [20]. Another reason for this enhanced antibacterial activity could be the high sensitivity of the *Bacillus* strain to medicinal plant extracts as compared to the *Pseudomonas* bacteria. A study revealed that gram-negative bacteria are less prone to antibacterial drugs as compared to gram-positive bacteria [21].

Among the bacterial strains, the T. *cordifolia* extract exhibited the largest zone of inhibition. This significant inhibition may be due to the presence of flavonoids or tannins in the extract, known for their antibacterial properties. Conversely, extracts showing smaller zones of inhibition might have limited penetration through agar, rendering them less effective in generating larger inhibition zones. This observation aligns with research suggesting that the antibacterial efficacy of medicinal plants could hinge on factors such as the solubility of antibacterial compounds in solvents or the migration properties of plant extracts to the surrounding agar, thereby inhibiting bacterial growth. [22].

Antioxidative assays of the selected plant extracts were performed to check their antioxidative abilities. The ethyl acetate extract of *T. cordifolia* showed maximum antioxidative properties except for the DPPH, in which *Q. infectoria* shows the highest antioxidative potential, i.e., (77%). In the Phosphomolybdate assay, the addition of molybdenum to the plant extracts causes its reduction into Mo (V). This reduction produced a green-colored complex. *T. cordifolia* extract showed maximum phosphomolybdate reduction activity (520 µg/ml). FRAP reduction occurred through the action of antioxidants. By this, ferrous ions get reduced to ferric ions. The antioxidants present in the extracts of *T. cordifolia* caused the reduction of Fe^{3+} complex to the ferrous form, and thus proved to have reducing power (371 µg/ml). *T. cordifolia* extract showed greater flavonoid content (170 µg/ml). This revealed that *T. cordifolia* has a high percentage of phenolic content, which makes it a good antioxidative agent. The increased antioxidative efficacy observed in specific plant extracts might be attributed to the presence of phenolic compounds within them. Phenols have the capability to function as free radical terminators, thereby amplifying the capacity for radical scavenging. Consequently, it could be inferred that the extract from *T. cordifolia* harbors a substantial proportion of phenolic content.

Table 2. GC-MS analysis of a partially purified bio active components of extract of *T. cordifolia*.

Compound name	Molecular formula	Molecular weight (g/mol)	Retention time	Structure
Benzene Acetic Acid	$C_{20}H_{40}O_5Si_4$	472.9	25.33	
Phthalic Acid	$C_{24}H_{38}O_4$	390.6	32.36	

This observation is consistent with the findings of Martin et al., suggesting that the presence of phenolic compounds can elevate antioxidative potential [23].

Various components present in the extracts were separated with the help of the thin-layer chromatography (TLC) technique. All of the components were separated into different bands depending on their solubility in the solvent system. A brown band was observed on the TLC plate after its treatment with iodine. In compliance with the study of Chavan and coworkers, the brown spot indicates the presence of sugars in the extracts [24].

GCMS analysis of the antibacterial activity possessing fraction of *T. cordifolia* extract was done. Two fractions were found to have antibacterial properties, but their activity was less than the activity observed by the whole plant. The reason behind this difference could be due to the loss of some bioactive fraction during extraction or the synergistic effect of various components in the whole plant as compared to single fractions. Another reason could be the irreversible binding of the components to the chromatographic resins [25]. GCMS analysis of these compounds has shown them to be Benzene acetic acid and Phthalic acid. Phthalic acid is reported to have antibacterial potential [26]. Benzene acetic acid or phenyl acetic acid also possesses antimicrobial activities, as suggested by recent studies [27]. The antimicrobial activities of these two compounds (phthalic acid and Benzene acetic acid) can be a beneficial source for future drug-related studies.

5. CONCLUSIONS

In conclusion, the study found that the ethyl acetate extract of *Tinospora cordifolia* (Giloy) exhibited significant antibacterial and antioxidant properties, with the presence of bioactive compounds such as Phthalic acid and Benzene acetic acid. These findings highlight the potential applications of *T. cordifolia* as a valuable medicinal plant for the development of future antimicrobial drugs. Future research should focus on structurally analyzing the phytochemical components of these plants with anti-dengue properties. The isolation and utilization of phytochemicals with anti-dengue properties hold promise for pharmacological applications.

6. ACKNOWLEDGMENTS

The authors are thankful to the University of the Punjab for the funding.

7. CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 189-198 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)862

Pakistan Academy of Sciences

Research Article

Geographical Information System, Remote Sensing and Multi Influencing Factors Techniques for Delineation of Groundwater Potential Zones in District Charsadda, Khyber Pakhtunkhwa, Pakistan

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Abstract: The effective harvesting of groundwater and its sustainable management is possible with the proper identification of zones on its potential basis. For this purpose, geographical information system (GIS) and Remote Sensing (RS) data approaches were combined and used for the investigation, preservation, and evaluation of groundwater supply. This study aimed to assess groundwater delineation and availability-based potential of groundwater area in Charsadda District, KPK, Pakistan, by applying the approach of RS, GIS, and multi-influencing factors (MIF). For this purpose, digital elevation model (DEM), shuttle radar topographic mission (SRTM), and sentinel 2 satellite images were employed to produce numerous thematic layers, i.e., land use land cover (LULC), drainage density, lineament density, geology, slope soil, and rainfall as MIF in this study. After assigning a fixed score and weight to each thematic layer (MIF techniques), then an analyzed spatial layer was combined with a weighted overlay using ArcGIS software (ArcMap 10.5) and finally potential area for groundwater was defined. The obtained potential area of groundwater was categorized spatially as, very high, high, moderate, and low zones which depict most of the area is covered with moderate (547.66 km^2) to high (306.7 Km^2), as well as, high highest groundwater potential in 7 km^2. The results of this study and the approach will be applicable and insightful for regional and extensive levels of developmental planning and harvesting of groundwater resources.

Keywords: Geographical Information System, Remote Sensing, Multi Influencing Factor, Groundwater Potential Zones, Weighted Overlay Tool.

1. INTRODUCTION

Water is vital resources on earth surfaces which form the foundation as of life [1]. It is present in different forms on the earth surface such as in ocean, glaciers, rivers, lacks, pounds, spring, as well as, underground water. Generally, the groundwater is mentioned for the water quantity present beneath the surface of earth. It is most vital natural resources on which the life of human depends to a great extent. Due to its importance the demand of groundwater for domestic and agriculture purposes is also increasing [2-4]. In perspective of groundwater as a fresh water source, it is now a constraining asset in major regions of the world which may grow further due to expanded population, urbanization and environmental changes. In perspective of largest fresh water source on earth, the potential based assessment of groundwater is crucial to conserve this resource [5]. Groundwater is also a key natural resource for a continuous provision of drinking water. At present, it contributes a major share in the total annual supply of water globally. Hence, sustainable for groundwater management systems, focused assessment for natural resource is very critical. In Pakistan, the groundwater resource is of critical importance due to its agrarian economy. Additionally, modern industrial, population as

Received: March 2023; Revised: May 2024; Accepted: June 2024
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well as agriculture have built-up pressure on groundwater and appropriate accessibility of surface water resources. Hence, there is a need for an applicable approach which is economically viable and gives reliable and time assessment of areas with sustainable groundwater potential. For this purpose, integration of Geographical Information System (GIS) and remote sensing (RS) is a reliable tool to determine and delineate the potential groundwater zones and extensively used for natural resource management [6]. In recent years, applications of satellite data coupled with mapping-based approaches has made it relatively convenient to ground based information for determination of groundwater potential zones [7-9]. Various studies were conducted on the identification and assessment of hidden potential zone of groundwater by applying integrated geospatial approaches and analytical hierarchy process (AHP) [10-14].

The studies with a focus on potential zones of groundwater has already been conducted globally which revealed that various factors which influence the determination of groundwater potential show variations, hence, in accordance, the final results also varied. Integrated studies using conventional data in integration with GIS tools and interpretation of satellite image data techniques are reliable as well as, economically viable and it increases the results of the accuracy [10-15]. Furthermore, it is extensively followed for characterization of the earth surface and assessment of groundwater recharge areas [3]. With combination of geospatial technologies (GIS and RS), potential area for groundwater have been delineated [6, 16]. Other related studies focused on merging of different related geophysical and hydrological factors [3, 17, 18]. These results are based on the field survey and are relatively reliable and varies to region basis due to different geoenvironmental conditions. In northwestern China a comparative study was carried out for simulating groundwater dynamic with applications of machine learning and numerical models, as well as for groundwater potential zone a combined approach of geospatial and geophysical was applied [19-23]. Various studies were conducted with a focus on delineating the groundwater potential for prospective planning using integrated multi-influencing factors (MIF), AHP, GIS modeling and geospatial approaches to find out the potential zones [24-30]. Integration of GIS and RS for preparation of assigned weightage base thematic layers in a spatial domain can help to find out the area of groundwater potential. This research aimed at finding out and demarcate the area of groundwater potential in the Charsadda District of KPK using an integrated approach of multi influencing factors, i.e., soil data, geology, lineaments data, rainfall data, drainage network, slope, Land use land cover (LULC), GIS and RS.

2. MATERIALS AND METHODS

2.1. Study Area

Charsadda District lies within Peshawar Division of KPK province, Pakistan. This area stretches from 34°-03′ to 34°-28′ N latitudes and 71°-28′ to 71°-53′ E longitudes. In the North it is surrounded by Malakand District, Mardan District by East, District Nowshera and Peshawar is on the South and Mohmand agency in the West as shown in Figure 1. On administrative basis, District Charsadddа has three tehsils that are Shabqadar, Charsadda and Tangi. It covers an overall area of 996 km^2 and is situated at an altitude of around 276 meters (906 ft) above sea level. The total population of Charsadda District is 1,835,504 (Census 2023).

In perspective of climatic aspects, summer season in Charsadda District remain hot from May to September. During summer, month of June remains relatively very hot with a temperature rise of over 40 °C. Monsoon season remains from July to September while months of July and August are hot and humid. The summer season changes in October and mid of February. A pleasant spring season starts from mid of the March. Highest summer rainfall prevails in the month of August while in winter, due to western disturbances, rainfall shows high record in the month of March and April.

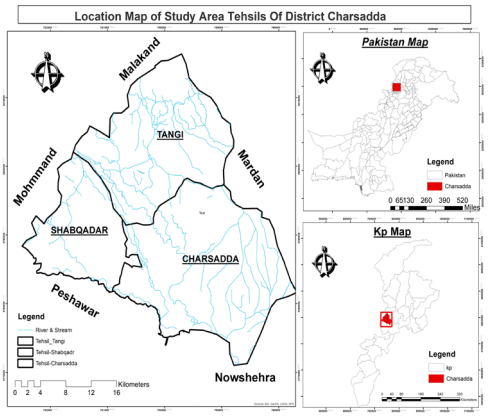

Fig. 1. Study area map.

2.2. Methodology

Weighted overlay analysis and multi influencing factor (MIF) approach are applied as methodology for this research. This approach is robust to retrieve factors which influence groundwater potential. This methodology comprises step wise approach as: the determination of parameters (variable) which have critical influence on groundwater potential. These critical factors are than used in this research to map out potential of groundwater in District Charsadda KPK, Pakistan including rainfall data, lineaments data, geology, soil data, slope, drainage network and LULC. Subsequent step was then weight and rank assignment to these parameters for ensuring uniformity. The final step was weighted overlay method in which all the determined data were reclassified for weighted overlay analysis. The output of the groundwater potential area and weighted overlay analysis is categorized as, low, moderate, high and very high. The flow methodology of present study is shown in Figure 2.

2.2.1. Determination of multi influencing factors (variables)

For the this study the variables which were applied as MIF to determine groundwater potential zones include: geology, soil, slope, LULC and drainage density.

2.2.2. Land use land cover (LULC)

Land use show that how much land is utilized by people for development, conversion and for other usages, i.e., area covered by agriculture, forest, water, built up and barren land. LULC analysis is very critical for groundwater study because the build-up area cannot hold water due to high runoff on that surface; while vegetation like forest, agriculture and plantation can trap and hold water through roots. For this purpose, a 10 m resolution image of sentinel-2 was processed to analyze the LULC. The method includes the process of supervised classification in GIS (ArcMap 10.5) environment. As a spatial output, the whole District was categorized into four different classes including water bodies, barren, vegetation and built up as illustrated in Figure 3.

2.2.3. Slope

Through slope analysis infiltration rate and runoff of surface water was determined. Gentle slope decrease runoff and increase infiltration into surface while steep slope expedites runoff water

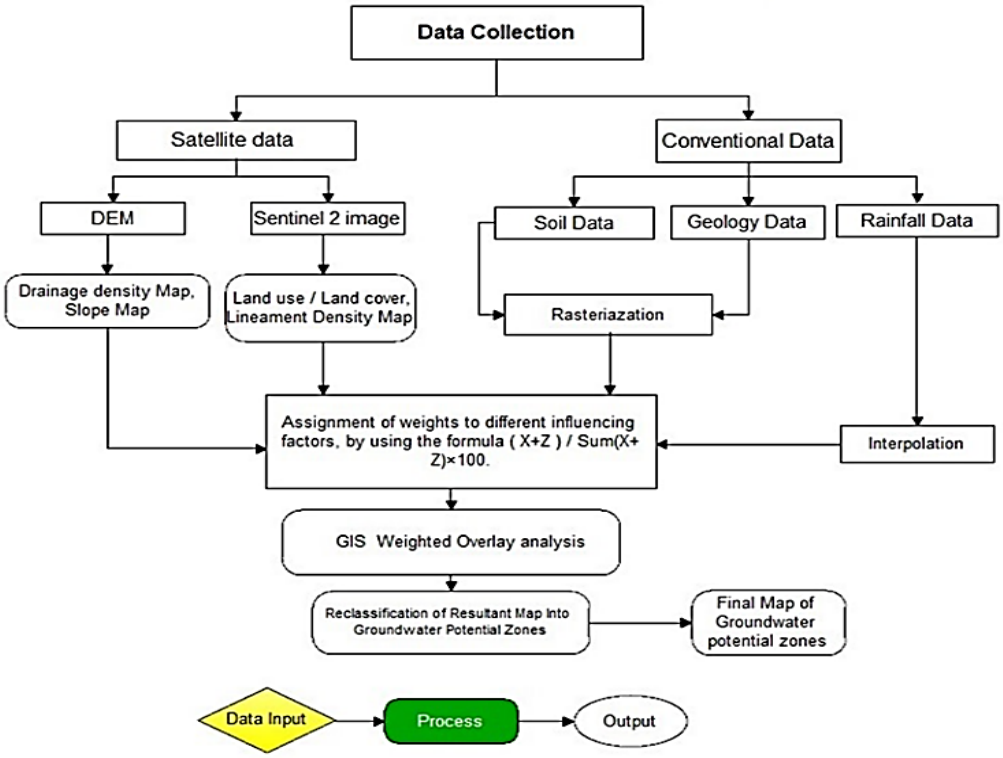

Fig. 2. Methodology flowchart.

and decrease water infiltration into ground. For this purpose, digital elevation model (DEM) and shuttle radar topographic mission (SRTM having 12 m resolution) were analyzed for the slope of study area through Arc Map.

2.2.4. Drainage density

Drainage density is also a critical factor due to its inverse function of permeability. The runoff of water will be high if the drainage density is more and resultantly penetration into ground will be lower while in low drainage density area the runoff going to be low and seepage into ground is more. For this study, drainage density was calculated from DEM data and SRTM with 12 m resolution in Arc Map 10.5 environment through built-in hydrology tools.

2.2.5. Rainfall

For detection of ground water potential based zones, rainfall is one of significant variables. The amount of rainfall due to which the atmospheric variation is not uniform in all places and varies spatially, as well as, temporally. For determining the influence of rainfall in any region, long time data-based study is necessary. When rainfall is high the volume of groundwater will be high, while in low rainfall area, the groundwater volume will also be resultantly low. For this study, the rainfall data of the years 2015 to 2019 was considered which was obtained from Peshawar meteorological station. The tabulated data was imported to ArcGIS environment and interpolated through 'Spatial Analyst' (built-in extension) to extract the spatial results.

2.2.6. Lineament density

Lineament, like a basic geological structure, can be easily identified on the ground. By lineament density, the subsurface faults and fractions, as well as, the presence of groundwater resources can be observed. High lineament density is the indicator of more groundwater while low lineament density means less groundwater. Hence, area with high lineament density have relatively high potential for groundwater. In this study, to extract lineament density, Sentinel 2 image obtained from European Space Agency was processed within PCI Geomatica (2018) software.

2.2.7. Geology

The surface geology determines the presence and distribution of groundwater. The level of groundwater (low or high) depends on the rock type. Rocks with high porosity have more water potential for water storage while low porosity rocks have less water storage capability. The water transfer from recharge area to discharge area during rock formation under the impact of hydraulic gradients is dependent on permeability. For geology of study area, the data was collected from existing geological map of Pakistan.

2.2.8. Soil

Ground and surface water infiltration depends on permeability and porosity of soil; therefore, it is considered one of the most critical factors for delineation of groundwater [6]. The study of soil is critical for determination of groundwater potential zones. To analyze the soil of area, soil map was digitized from soil data which was collected from soil conservation department.

2.2.9. Weight and rank assignment to parameters

For the present study, we selected seven factors such as lineament density, drainage density, rainfall, slope, soil, geology, and LULC, for delineation of groundwater potential zones has diverse influence towards the groundwater. For that purpose, weight of each factor was given depending on its effect to the storage and movement of water [3, 18-31]. Proposed weight of each influencing factors was found by the equation (1) as follows:

$$\frac{X+Z}{\Sigma(X+Z)} \times 100 \quad (1)$$

While, X show major effect while Z show minor effect of factors. Total weightage of each parameter was equally divided among the sub-classes of each parameter. The weigh was assigned to each-sub class between 9 to 1, where 1 shows very low or no groundwater while 9 shows area with possibility of high groundwater concentration [32-36].

2.2.10. Weighted overlay method

The MIF data was subsequently reclassified for weighted overlay analysis. Reclassification was done through ArcMap spatial analysis tool in Arc

GIS. After assigning weightage and ranks, the overlay method was applied on reclassified data through weighted overlay analysis tool in Arc Map. The output of the weighted overlay analysis was resultan spatial product of groundwater potential based zones.

3. RESULTS AND DISCUSSION

3.1. Spatial Results of the Multi Influencing Factors (MIF)

3.1.1. Land use land cover

Supervised classification method was used for LULC mapping acquired from sentinel 2B [37]. Supervised classification image based LULC identification was classified into four classes namely: vegetation, settlement, water body, and barren. Based on its spatial results, most of the area is covered by agriculture. High weightage is given to the spatial class of 'water bodies' as these are excellent resource for groundwater recharge as shown in Figure 3.

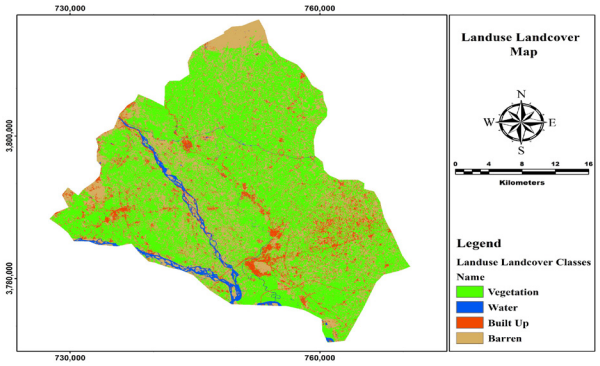

Fig. 3. LULC map.

3.1.2. Slope

Slope map was generated from DEM and categorized as suitable, most suitable, moderate, less suitable and not suitable [37, 38]. Infiltration of water is inversely proportional to runoff on the surface water which determine slope. When slope is increased, water runoff will be increased and vice versa. The slope map (in degree) was spatially classified into five classes: flat (0-2.34°), gentle (2.34-5.39°), moderate (5.39-12.18°), steep slope (12.18-24.37°) and escarpment (24.37-59.75°) as shown in Figure 4. The study area has averagely plain surface (flattened). Therefore, it increases the holding and drainage of water inside ground which resultantly leads to less runoff of water and its more infiltration.

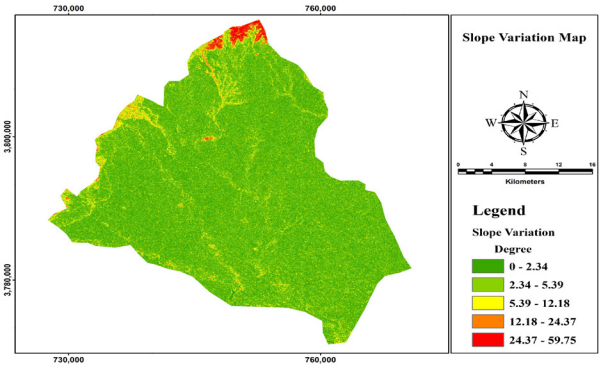

Fig. 4. Slope map.

3.1.3. Drainage density

High drainage density area has low rate of recharge, while area having low drainage density has better potential for groundwater recharge rate due to its inverse relation of groundwater. In previously studies, the drainage density for Islamabad and Rawalpindi was investigated and the map was categorized into five classes [37, 39]. Area of the drainage density extracted from DEM data was spatially categorized into five density classes having values 1-1.8 Km^2 is very low, 1.8-2.6 Km^2 is low, 2.6-3.4 Km^2 is moderate, 3.4-4.2 Km^2 is high and 4.2-5 Km^2 is very high. Figure 5 shows that the area having low drainage density has better potential of groundwater due to less stream and rivers which mean less surface runoff water.

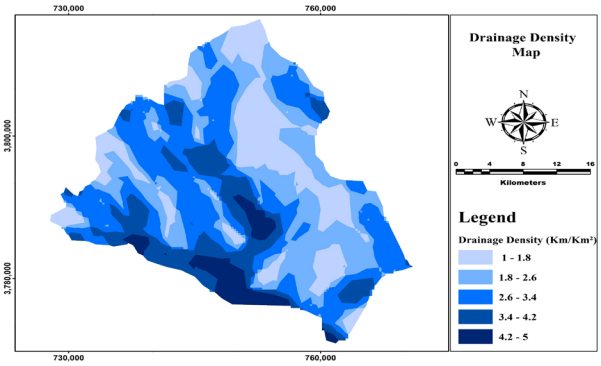

Fig. 5. Drainage density.

3.1.4. Rainfall

Interpolation method was used to acquire the monthly basis rainfall maps for different years [38].

Rainfall map (generated from rainfall data of five years: 2015-2019) was reclassified into five spatial based classes as: very low (15-22 mm), low (22-30 mm), moderate (30-37 mm), high (37-44 mm), and very high (44-51 mm) as show in Figure 6. The spatial results reveal that average annual rainfall of the area ranges from 15.7 mm up to 51.4 mm.

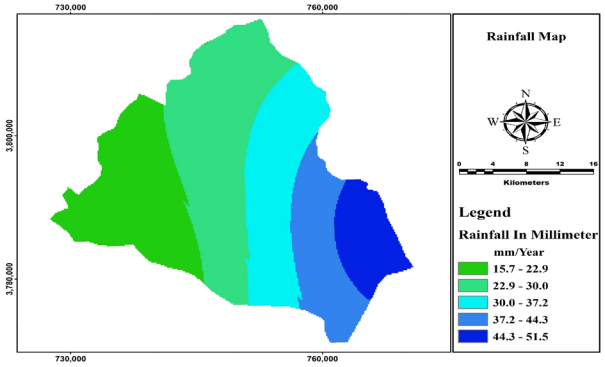

Fig. 6. Rainfall map.

3.1.5. Lineament density

The high density of lineament encompasses numerous faults which are not suitable for storage of water. Lineament disturbs groundwater recharge, surface storage and flow [40, 41]. The lineament density was spatially categorized as: very low (0-0.1 Km^2), low (0.1-0.2 Km^2), moderate (0.2-0.3 Km^2), high (0.3-0.4 Km^2) and very high (0.4-0.5 Km^2). In this study, areas with lineament density between 0.4 and 0.5 km/km^2 were considered best for prospective groundwater zones. According to its spatial results in map, it is clear that most parts of this area comprise very poor lineament density (Figure 7). Area with low lineament was assigned low weightage value while area having high lineament was assigned high weightage value.

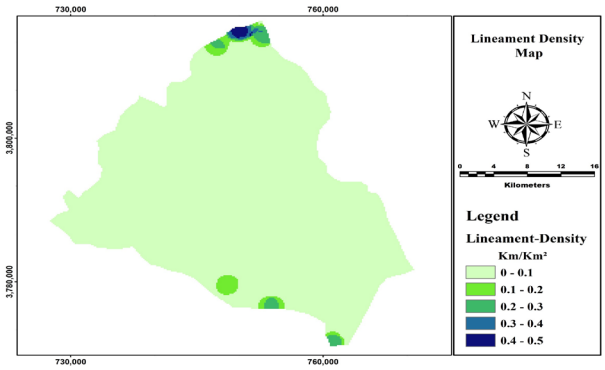

Fig. 7. Lineament density.

3.1.6. Geology map

The characteristics of geology, in effect, control the subsurface aquifer to the transfer of groundwater from up-surface stream and the divergent movement [38]. Surface geology is the controlling factor of flow and infiltration quantity of the groundwater. The research area comprises Mesozoic Meta, Quaternary Alluvium and Mélange rocks. Alluvium is unconsolidated and loose rock that can easily erode and reshape by water. For the study area, high weightage was assigned to alluvium rocks because it has high infiltration rate as compared to other rock types in the focus area as illustrated in Figure 8.

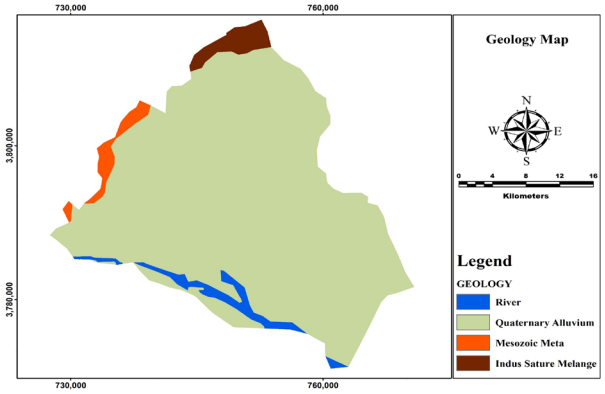

Fig. 8. Geological map.

3.1.7. Soil map

Previously Alam *et al*. [37] divided the soil map into five classes based on the permeability ratio. The study area comprise many soil types which includes loams, loamy sands, silt loams, silty clay loam and silty clays. The statistical study of the study area depicts that most part is covered by silt loams which has low penetration rate (Figure 9). The high ranks values were assigned to loamy sands and loams which are good for groundwater.

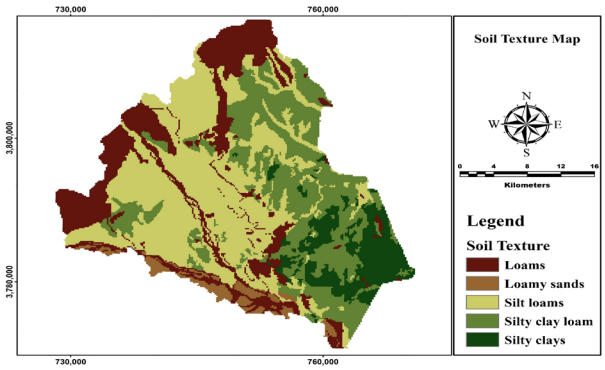

Fig. 9. Soil map.

3.1.8. Results of the influencing factors

Table 1 represents sub classes of each variable with each class influence towards groundwater, as well as, rank of each class. While, X represent major effect and Z represent minor effect of these MIF (seven factors selected for the present study).

3.1.9. Ground potential zones

The potential of groundwater in the focus area in perspective of its spatial distribution follow the regional influencing pattern of LULC, drainage density, lineament density, rainfall, as well as, physiological factors include geology, soil, and slope. Based on ground potential zone, the spatial output was classified as low, moderate, high and very high, respectively (Figure 10). Overall, moderate groundwater potential area is identified in Figure 10. Resultantly, major area is covered with zone of moderate, very high and high area of 7 km^2 and 306.71 km^2, respectively, while the low potential zones cover relatively less area of 105.91 km^2 as illustrated in Figure 11.

Table 1. Weightage of different factors.

S. No.	Parameters	Major effect (X)	Minor effect (Z)	Proposed relative effect (X + Z)	Proposed weight [Equation 1] (rounded figure)*
1	Rainfall	1 + 1	0.5 + 0.5 + 0.5	3.5	17.5 (18)
2	Geology	1	0.5 + 0.5	2	10
3	Slope	1	0.5 + 0.5 + 0.5	2.5	12.5 (13)
4	Soil	1 + 1	0.5 + 0.5 + 0.5	3.5	17.5 (17)
5	Lineament density	1	0.5 + 0.5 + 0.5	2.5	12.5 (12)
6	Drainage density	1 + 1	0.5 + 0.5	3	15
7	Land use/Land cover	1 + 1	0.5 + 0.5	3	15
	Total			Σ20	Σ100

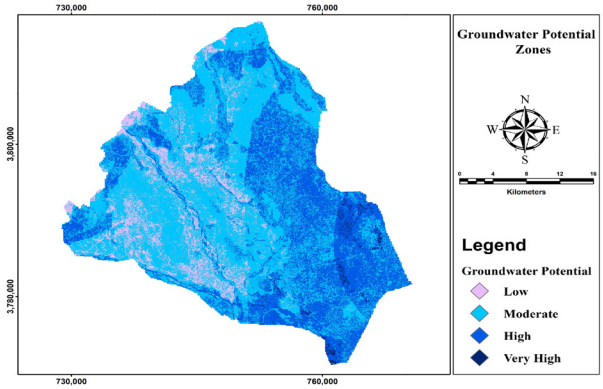

Fig. 10. Zones of groundwater potential.

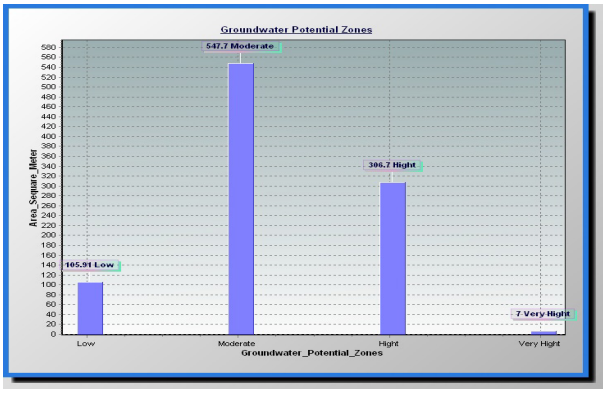

Fig. 11. Graph of zones with groundwater potential.

4. CONCLUSIONS

This study adopted the GIS, RS and MIF based techniques and approaches which are relatively reliable and applicable technologies-based methodology for delineating groundwater potential based zones. Overall, analysis of seven critical influencing parameters including geology, slope, soil, lineament density, drainage density, LULC and rainfall were carried out as MIF from RS data, GIS environment to access groundwater potential zones of district Charsadda. Finally, results of groundwater potential based zones were delineated into classes as Low, moderate, high, and very high zones (Figure 10). The resultant data reveal that this area is mostly covered with zones of relatively moderate groundwater potential zone. The 'high' potential zone cover area is 7 Km^2 while zone with 'very high' potential covering the area of 306.71 Km^2 considered as zones with high potentiality of groundwater. Overall, the low potential zone covers relatively less area of 105.91 km^2. Hence, moderate

to highest potential zones will be helpful for usage of groundwater. Prospectively, this study approach may be efficient for applicable management and eco-friendly development of groundwater supply.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 199-205 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)881

Pakistan Academy of Sciences

Research Article

Physico-Chemical Properties, Phytochemical Screening and Antioxidant Potential of Polar and Non-Polar Seed Extractions of Selected Medicinal Plants of *Solanaceae*

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Abstract: The objective of this study was to investigate the various bioactive components and assess the biological activity in the seeds of five medicinal plants (*Datura innoxia* Miller., *Solanum elaeagnifolium* Cav., *Solanum nigrum* L., *Solanum surattense* Burm., and *Withania coagulans* (Stocks) Dunal.) in *Solanaceae* family. In this regard, ash values, acid-insoluble ash and water-soluble ash values, extractive values and screening of various phytochemicals (alkaloid, carbohydrate, protein, flavonoid, tannins, saponins, and fixed oil) and quantitative estimation of carbohydrate and protein of seeds of selected plants have been calculated. The 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging assay was used to evaluate the antioxidant potential. Captivatingly, a phytochemical screening assay affirmed the presence of saponins, terpenoids, phenolic compounds, and flavonoids in the seeds of all examined plants. The highest amounts of DPPH radical-scavenging activity were found in the aqueous extract of *W. coagulans* (IC_{50} = 65.6 µg/ml). Consequently, current findings showed all selected plants of the family *Solanaceae* contain highly bioactive components, which could be used for the treatment of different diseases and as a potential resource for discovering new drugs.

Keywords: Medicinal Plants, Bioactive Components, *Solanaceae* Family, Phytochemical Analysis, Quantitative Estimation, Antioxidant Activity.

1. INTRODUCTION

Medicinal plants have been employed in healthcare systems from the beginning. Studies have been done all around the world to verify their efficacy, and some of the findings have spurred interest in the production of plant-based medications [1]. As per the World Health Organization, 80 percent of the world's population takes plant-based treatment as their primary form of health care and about 11% of the 252 drugs are exclusively derived from plants. In modern society regardless of the advancement of synthetic drugs plants are the fundamental source of new healthcare and pharmaceutical products [2]. The majority of research supports the effectiveness of medicinal herbs, which has sparked interest from all over the world. Medicinal plants offer therapeutic benefits and have fewer negative side effects than synthetic drugs [3].

Depending on their biological roles, the phytochemicals that plants generate might be classified as primary or secondary metabolites. Their primary elements include common sugars, proteins, amino acids, purines, and pyrimidines from nucleic acids, as well as other materials like chlorophyll. Secondary components encompass the remaining plant compounds, which include alkaloids, phenolics, glucosides, saponins, terpenes, lignans, flavonoids, and plant steroids [4]. Although secondary metabolites are produced in all part of the plant, including the bark, stems, leaves, roots, fruits, flowers, seeds, and so on, plants may not require them. These substances are thought to be therapeutic mediators and effective in treating a wide range of illnesses in both people and animals. exhibit a variety of significant pharmacological properties, including antibacterial, antiviral, antirheumatic, cholesterol synthesis inhibition, anticancer, and antiparasitic effects [5].

Plant species have strong antioxidant activity, which could be used to find effective treatments for tissue damage caused by free radicals. Various

Received: March 2023; Revised: May 2024; Accepted: June 2024
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synthetic antioxidants, such as tertiary butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), have been incorporated into food products. However, these artificial antioxidants have been linked to liver problems [6]. With 84 genera and 3000 species, the *Solanaceae* family is extensively distributed in tropical and temperate regions of both hemispheres, but primarily in Western and Southern America. It is represented in Pakistan by 14 genera and 52 species. 27 species are native, 6 naturalized and others are cultivated and found rarely as escapes [7].

Various plants of the *Solanaceae* family have been traditionally used as medicine all over the world. It is because of the presence of various secondary metabolites. These are the most potent known anticholinergics, which means they suppress the neurological impulses provided by the endogenous neurotransmitter acetylcholine [8]. It has been reported that *Solanaceae* plants, including *Capsicum*, *Datura*, and *Solanum*, contain anti microbial peptides (AMP) and peptide-rich extracts from their seeds, leaves, fruits, and tubers. Notable antifungal, antimicrobial, or antiviral effects against human pathogenic microorganisms and phytopathogenic strains have been reported for these peptides. *Datura stramonium* L. seed extracts have been reported to have antibacterial activity against *Escherichia coli* and *Klebsiella pneumonia* [9]. It has been demonstrated that an extract from the leaves of *Solanum lycopersicum* shows potential antitumor properties against breast cancer cells by modifying the expression of genes linked to the development and spread of cancer. *Withania somnifera* leaf extract activates tumour suppressor proteins and has cytotoxic effects on human osteosarcoma, fibrosarcoma, and lung cancer epithelial cells [10].

The focus of this study was to investigate the seeds of five different plants from the *Solanaceae* family that were taken for physicochemically, phytochemically, and free radical scavenging ability analysis, i.e., *Datura innoxia* Miller., *Solanum elaeagnifolium* Cav., *Solanum nigrum* L., *Solanum surattense* Burm., and *Withania coagulans* (Stocks) Dunal. This research will aid in the verbalization of the phytochemical standard. As a result, basic knowledge regarding phytochemicals will be important in determining therapeutic effectiveness.

2. MATERIALS AND METHOD

2.1. Collection and Sample Preparation

An appropriate amount of seeds (150 g) of selected medicinal plants were collected from different areas of Karachi, Pakistan, sourced from their natural habitats (wild). With the seeds collection, plant specimens were also collected for identification purposes. Identified plants (Herbarium sheets) were deposited in S.I. Ali Herbarium, Center for Plant Conservation, University of Karachi. The seeds were dried at room temperature. Clean and well-dried seeds (100 g) of each plant species were blended into a fine powder using an electric blender.

2.2. Preparation of Crude Extracts

20 g powdered sample was soaked in 200 ml of different solvents, *viz.,* acetone (99.5%), chloroform (99.8%), methanol (99.8%) (BDH Laboratory Supplies), and water, separately, and placed for 48 hours for continuous shaking in an orbital shaker. After 48 hours of continuous shaking, the extracts were filtered with the help of Whatmann No. 1 paper and left for evaporation until all the solvent had evaporated. The dried material was collected, and the extractive value was calculated by weighing the sample with the help of an electrical weighing machine (Sartorious TE214S).

2.3. Physicochemical Analysis

Physicochemical analysis was carried out such as moisture content as per the standard method. At 105 ºC, 2 g crude powder of plant seeds was dried, weight was noted, drying loss was computed, and percentage was determined based on the initial sample [11]. Total ash content was calculated, 2 g of plant seed powder were ignited in a crucible at 500 °C until it turned white, signifying the absence of carbon. The ash was then rapidly cooled and weighed. The percentage of ash was calculated based on the initial sample weight. Additionally, the physical state, color, and ash values (water-soluble ash and acid-insoluble ash) were determined according to standard methods [12, 13].

2.4. Qualitative Phytochemical Analysis

For the phytochemical assessment, standard methods were employed. Alkaloids were identified

using Mayer's test, Wagner's test, and Hager's test [14, 15]. Carbohydrates were detected through Benedict's test and Fehling's reagent [16]. Proteins and amino acids were assessed using the Biuret test and Millon's test [17]. Phenolic compounds were identified by the Lead acetate test and Ferric chloride test [18]. Fixed oils were evaluated using the spot test, and saponins were detected through the Foam test. Glycosides were assessed using Borentrager's test [14].

2.5. Quantitative Estimation of Carbohydrate and Protein

The total carbohydrate of seeds was estimated using Anthrone reagent [19]. 4 ml of the anthrone reagent was mixed with 1 ml of the sample. After 10 minutes of incubation in a boiling water bath, the absorbance at 620 nm was measured with the spectrophotometer in comparison to a reagent blank. The results were reported as mg/g sample, and the estimation was carried out in triplicate.
The Bradford assay was used to determine the total protein content by mixing 3 ml of Bradford reagent with 0.1 ml of the sample. Following an incubation period of 10 minutes at room temperature, the absorbance at 595 nm was measured using spectrophotometer [20].

2.6. Determination of Antioxidant Activity

The approach utilized to test the antioxidant activity was based on an assessment of the extract's free radical scavenging activity using the method outlined by Brand-Williams *et al.* [21] with some modifications. The DPPH scavenging activity of different extracts of acetone, chloroform, methanol, and water were determined. As a blank and standard solvent, methanol and ascorbic acid were utilized, respectively. From each extract, stock solutions (2.5 mg/ml) were produced in methanol. These stock solutions were used to make working solutions (50, 100, 150, 200, and 250 µg/ml). 1 ml of DPPH solution (0.96 mM) was added to each working solution before incubating for 30 minutes at room temperature. 1 ml DPPH was added to 4 ml methanol to make the control. The absorbance of the samples and the control was measured using a UV/VIS spectrophotometer at the 517 nm range. The DPPH scavenging activities were calculated as a percentage of inhibition using the equation:

$$\%\, inhibition = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of the sample. The IC_{50} is a measure of a compound's effectiveness in suppressing a particular function. The IC_{50} of the DPPH radical scavenging activity of seed extracts from selected plants was determined through linear regression analysis of concentrations and percent inhibition.

2.7. Statistical Analysis

All analyses were carried out in triplicate, with the results statistically analyzed and expressed as the mean (n = 3) and standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1. Physicochemical Analysis

Physicochemical analysis of seed powder of selected medicinal plants is shown in Table 1. The loss on drying of powdered seeds was observed at 12% in *D. innoxia*, 2.5% in *W. coagulans,* 7% in *S. elaeagnifolium,* 3.9% in *S. nigrum, and* 14% *in S. surattense.* The total amount of ash was highest for *W. coagulans* seeds, i.e., 12.5%; whereas the lowest was observed in *S. elaeagnifolium,* i.e., 5%. The highest water-soluble ash was found 5.21% in *S. elaeagnifolium* and the lowest 3.91% in *S. surattense.* The acid-insoluble ash was highest

Table 1. Physicochemical properties of seeds of selected medicinal plants.

Parameter	*D. innoxia*	*W. coagulans*	*S. elaeagnifolium*	*S. nigrum*	*S. surattense*
The physical state of ash	Granulated	Granulated	Granulated	Fine powder	Fine Powder
Color of ash	Brown	Black	Grayish white	Light brown	Creamy white
% loss on drying	12	2.5	7	3.9	14
Ash content (%)	6	12.5	5	7.88	6.5
Water soluble ash	4.2%	4.37%	5.21%	4.73%	3.91%
Acid insoluble ash	2.5%	3.21	4.1	3.9	0.5415

for *S. elaeagnifolium,* i.e., 4.1% and the lowest for *S. surattense,* i.e., 0.54%. Extractive values of selected medicinal plants seeds of *Solanaceae* in different solvents are shown in Figure 1. The highest extractive value was observed in chloroform extract of *W. coagulans, S. elaeagnifolium, and S. surattense.* The highest extractive value of seeds of *S. nigrum* was found in methanol and lowest in acetone. The solubility of *D. innoxia* seed extract was highest in water and lowest in chloroform.

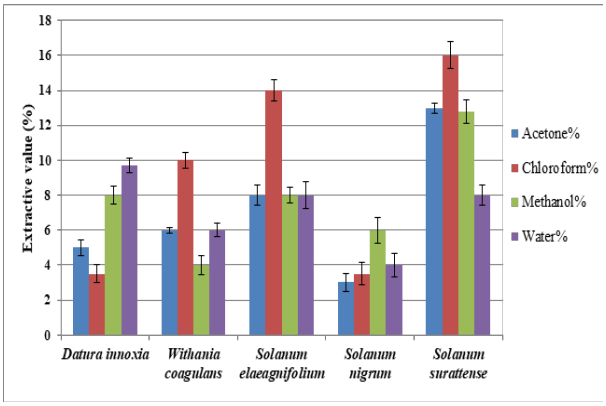

Fig. 1. Extractive values of seeds of selected medicinal plants in four different solvents.

3.2. Phytochemical Analysis

Historically members of the family *Solanaceae* were highly valued for their alkaloid content, which was utilized for poisoning and psychotropic effects; however, a study of the bioactive chemicals of this family is now becoming more important for medicinal applications [22]. The leaves of *Solanum torvum* have been reported to possess antimicrobial activity [23]. An ethanolic extract of *Datura stramonium* leaf showed a considerable anti-inflammatory effect in rats with carrageenan-induced paw edema [24]. Extracts of *S. nigrum* are analgesic and anti-inflammatory [25]. Ramadan *et al.* [26] reported that the seeds of *Datura innoxia* contain fatty acids and fat-soluble compounds. Pharmacological screening of the plant extracts

Table 2. Qualitative analysis of seeds of selected medicinal plants of family *Solanaceae* in four solvent extracts.

	D. innoxia				*W. coagulans*				*S. elaeagnifolium*				*S. nigrum*				*S. surattense*			
Solvent Extract	**Ac**	**Chl**	**Met**	**W**	**Ac**	**Ch**	**Met**	**W**	**Ac**	**Chl**	**Met**	**W**	**Ac**	**Chl**	**Met**	**W**	**Ac**	**Chl**	**Met**	**W**
Alkaloid																				
Wagner's reagent	+++	+++	+++	++	-	-	+	+	+++	+	+++	+++	-	-	+	+	-	-	++	++
Mayer's reagent	+++	+++	+++	++	+	+	+	+	+++	++	+++	+++	-	+	+	+	+	++	+++	+++
Carbohydrate																				
Benedict's test	++	++	+++	++	-	+	-	-	+++	+++	+++	+	+	-	+	+	++	+++	++	+
Fehling test	++	++	+++	++	+	-	+	+	++	++	++++	+++	-	+	+	+	++	+++	++	-
Protein																				
Biuret test	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	++	+++	+++
Millions test	+	-	++	++	++	++	+	+	++	++	++	++	++	++	++	++	++	++	++	++
Phenolic Compounds																				
Lead acetate	++	++	++	+++	+	+	+	+	++	-	++	+++	++	++	++	++	++	++	+++	+++
Ferric chloride test	++	++	++	++	+	+	+	+	++	-	+	++	++	++	++	++	+	++	+++	+++
Flavonoids	++	++	++	++	-	-	-	-	+++	+	++	++	+	+	+	+	-	+	+++	+++
Glycoside																				
Salkowsk's test	+	-	+++	+++	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
Terpenoids	++	+++	++	++	+	+	+	+	+++	+	++	+	+	++	++	++	++	+++	+++	+++
Fixed Oil																				
Spot test	+	++	++	+	-	-	-	-	++	++	++	-	-	-	-	-	+	++	++	++
Saponin																				
Foam test	+++	+	++	+++	-	-	-	+	-	-	-	-	-	-	+	+	-	-	+	+++

Key: Ac = Acetone, Chl = Chloroform, Met = Methanol, W = Water, + = present, - = absent, ++ = present in moderate amount, +++ = present in high amount.

provided insights into both their beneficial and toxic properties. Phytochemical investigation of selected medicinal plant seed extracts in various solvents revealed the presence of alkaloids, carbohydrates, proteins, phenolic compounds, flavonoids, terpenoids, fixed oil, and saponins (Table 2). These Phytoconstituents are considered remedies for a variety of ailments in both humans and animals. Alkaloids are present in all 5 selected plants, and have been attributed to medical use. Alkaloids are associated with cytotoxicity, analgesic, and antibacterial properties [27]. The present results show that most plants contain carbohydrates, except for *Withania coagulans*, which exhibited weak indications of carbohydrates in all solvent extracts of its seeds. Carbohydrates provide energy while also aiding digestion and nutritional absorption. Furthermore, some of the carbs in these plants have therapeutic properties. All five selected medicinal plants showed positive results for flavonoids. Flavonoids have strong anticancer activity and also help in managing diabetes and oxidative stress [28]. Flavonoids offer protection from hepatotoxins, tumours, viruses, and Alzheimer's disease. They possess antioxidant, antifungal, anti-carcinogenic, hepatoprotective, and cytotoxic properties [29]. The preliminary screening tests for phytochemicals can be helpful in exploring their bioactive values, which could lead to the discovery and development of new drugs [30].

3.3. Quantitative Estimation of Carbohydrate and Protein

The total carbohydrate content of various medicinal plants was assessed, and it was revealed that the maximum carbohydrate content was found in the aqueous extract of *Solanum elaeagnifolium,* i.e., 8.32 ± 0.11 mg/ml, and minimum carbohydrate content was obtained in the acetone extract of *Withania coagulans,* i.e., 0.68 ± 0.01 mg/ml (Figure 2). It was found that the protein was present in all of the plants studied in this investigation. The highest protein concentration was observed in the methanolic extract of seed of *Solanum surattense* 1447.68 ± 8.3 µg/µl and the lowest was present in the chloroform extract of *Datura innoxia* 57.43 ± 5.8 µg/µl (Figure 3).

3.4. Antioxidant Activity

The antioxidant capacity of extracts obtained from different solvents was investigated in this work utilizing DPPH scavenging activity tests. As shown in Figure 4 different extracts possessed varying free-radical scavenging activities. The IC_{50} value of antioxidants with strong scavenging ability should be low. Among the extracts analyzed antioxidant activity with different solvents varied IC_{50} value of 65.6 to 123.7 µg/ml after 30 min. The highest antioxidant value was observed by *W. coagulans* in the range of 65.62 µg/ml - 123.78

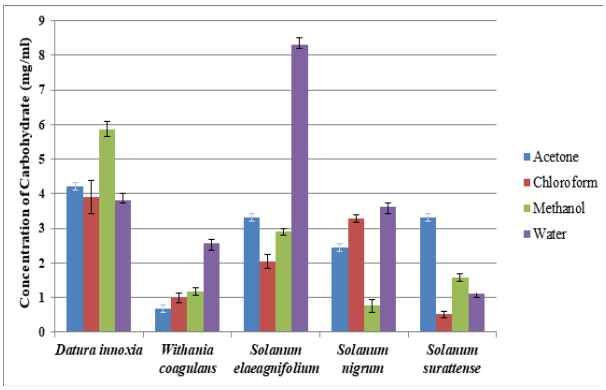

Fig. 2. Quantitative estimation of Carbohydrate in seed extract of selected plants

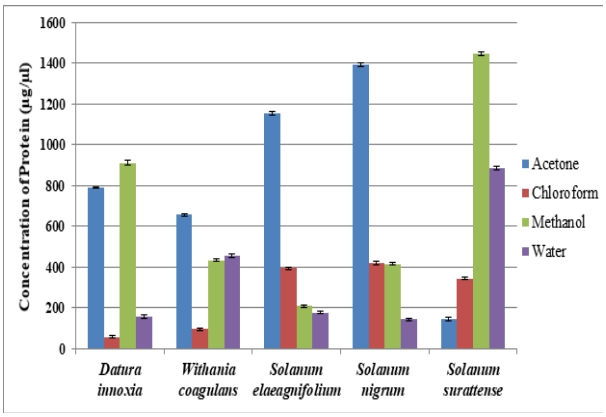

Fig. 3. Quantitative estimation of Protein in seed extract of selected plants.

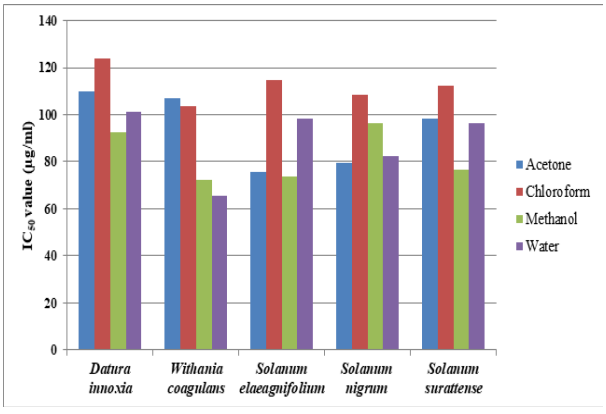

Fig. 4. IC_{50} values of DPPH free radical scavenging activity of seed extracts of selected *Solanaceae* species.

µg/ml, followed by *S. elaeagnifolium* in the range of 73.87 µg/ml -114.65 µg/ml, followed by *S. surattense* in the range 76.62 µg/ml - 112.5 µg/ml followed by *S. nigrum* in the range 79.5 µg/ml - 108.3 µg/ml, followed by *D. inoxia* 92.34 µg/ml - 123.78 µg/ml. Previous research has shown that changing the polarity of the solvent can be used to selectively concentrate antioxidant chemicals [31]. The presence of alkaloids and phenols in plant extract is often linked to its high activity, as these groups of phytochemicals include the bulk of active antioxidant substances. Secondary metabolites such as phenol and alkaloids play an important role in increasing antioxidant capacity [32]. Phenolic components are beneficial electron donors because their hydroxyl groups can contribute to the antioxidant process. Total phenol and antioxidant capacity have a strong positive correlation [33]. These findings could be utilized as markers for identifying and standardizing of the drug as an herbal treatment, as well as in the development of a monograph for the plant. Significant differences in free radical scavenging capabilities were observed in the current investigation for seed extracts of selected plants depending on the kind of extract utilized. The greatest scavenging capacity was demonstrate by aqueous and methanol extracts, followed by acetone and chloroform extracts.

4. CONCLUSIONS

The seeds of selected medicinal plants, due to their various bioactive substances, could potentially treat diseases such as rheumatism, diuresis, viral infections, cancer, malaria, fungal infections, and bacterial infections. Given their effects on human health, switching from synthetic to natural antioxidants could be advantageous. According to our findings, seeds of selected medicinal plants have the potential to be a source of valuable pharmaceuticals. Phytochemical screening of medicinal plants is important for both research institutes and pharmaceutical companies in the industrialized of the new drug.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 207-215 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)941

Pakistan Academy of Sciences

Research Article

Exploring Caregiver Burden of Thalassemia Major Patients

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Abstract: Thalassemia requires lifelong therapy that continuously strains patients, their families, and the local health system. This study was designed to analyse the challenges faced by carers and the effect they have on their social, psychological, and personal lives. Two hundred parents (100 cases and 100 controls) were recruited. In addition to socioeconomic status and concerns, feelings about children and their state of depression were also recorded by the PHQ-9 instrument. Statistically significant correlations were found which showed that parents are exposed to trauma at a very early age which limits their ability to become effectual individuals. Therefore, training and counselling must be worked out with extended family members for whom the health care body should also play its part in alleviating their sufferings. Overall, this study emphasized the significance of considering caregiver worries regarding improving the well-being of children.

Keywords: Thalassemia, Depression, Genetic Counselling, Extended Family Screening, PHQ-9.

1. INTRODUCTION

Thalassemia is the most prevalent monogenic illness, with an approximately 270 million carriers of hemoglobinopathies worldwide [1]. It is characterized by persistent anemia, hepatosplenomegaly, bone abnormalities, particularly of the facial bones, and an abnormal growth rate. Iron chelation therapy and routine blood transfusions become necessary for lifelong administration [2]. Due to absence of any proper registry for thalassemia in Pakistan, exact carrier rate is unknown but various reports have shown a carrier rate of approximately 5-7% [3, 4]. Unfortunately, among the WHO member states in the Eastern Mediterranean Region (EMRO), Pakistan had the greatest risk of thalassemia [5]. Consanguineous unions, huge populations with high birth rates, and ineffective preventive efforts are all regarded as major threat variables. A lack of awareness is also linked with high risk, which is dictated by illiteracy, social as well as cultural structure and religious preferences [2]. In modern society, parents are frequently obligated to provide for their children's needs, from blood transfusions to pharmaceuticals. The difficult routine and seeing a child in declining health have a negative impact on the caregiver [6]. The perceived impact of caregiver tasks on the caregiver's own sentiments and possessions is known as caregiver burden. It is a multifaceted concept that includes objective burden, subjective demand burden and subjective stress burden. While subjective demand burden refers to the interpersonal burden between carer and care recipient, objective burden refers to the interference with personal and social life. The emotional strain that the caregiver feels as a result of societal intolerance, self-blame for bad kid circumstances, improper responses, and reluctance to partake in social activities is known as the subjective stress burden [7].

The parents' primary concerns include their children's suffering, financial insecurity, and fear for their children's future [8]. As a result, understanding the causes of poor quality of life will aid in the development of appropriate methods of parent counselling. However, people use

Received: July 2023; Revised: May 2024; Accepted: June 2024
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various coping mechanisms to manage their stress. However, further research is needed in this area to uncover difficulties underlying the failure of coping techniques [7, 9]. All difficulties experienced by caregivers' in caring for a thalassemic kid are influenced by a variety of factors and variables. The hidden facets of these experiences can be explored by employing the conventional questionnaire-based study. In this regard, this study was designed to analyse their experiences caring for the patient as well as their perceptions regarding sickness.

2. METHODOLOGY

2.1. Subject Selection

For this case-control study, subjects were enrolled by a non-probable convenient sampling method from December 2012-13. Only parents who took their children to transfusion centres on a regular basis were asked to participate in this study on weekdays between the hours of 9 a.m. and 4 p.m. The following criteria were required for inclusion: (1) parent of no less than one kid with thalassemia, (2) registration at the corresponding transfusion centre, (3) willingness to contribute, and (4) neither a physical nor a mental defect. Exclusion criteria included: (1) being a family member or guardian of a kid with thalassemia, (2) not being completely involved or oriented in child therapy, (3) being unwilling to participate, and (4) having a depressive condition, physical or mental abnormalities, or prolonged mental health condition. An informed consent was obtained after giving a brief summary about study objectives and guaranteeing the confidentiality of provided information. Participations were offered no compensations and they can voluntarily discontinue at any time. One hundred cases at transfusion centre and 100 cases at home as control were enrolled. Among 200 subjects, 21 unfinished questionnaires and 04 voluntary terminations cut down the number to 175 including 83 cases and 92 controls. Data were collected over the course of 15-20 minutes depending on each participant's response at the transfusion centre for cases and at home for controls.

2.2. Data Collection

The information was obtained in three parts: the PHQ-9 instrument (9 questions about depressive indicators), psychologically suitable skills (especially emotion, data, as well as sustenance), along with sociodemographic information. Five mothers were shown the questionnaire with thalassaemic children in order to ensure that it was obviously implied. The questionnaire was prepared more comprehensible by detailing in Urdu language whenever possible. The PHQ-9 questionnaire was used to assess depression severity. It utilizes the contents of the Diagnostic and Statistical Manual, Fourth Edition (DSM-IV) [10], major depressive disorder diagnostic criteria. It has nine questions, and the findings were classified on a 4-point Likert scale from 0 to 1, reliant on the length of the indicators that had lasted during the previous one month. Data were encoded as: 0 = not at all, 1 = several days/months, 2 = half days/month and 3 = daily. Depending on the scores, the intensity of depression was rated into 5 rankings calculated such as: 1-4 = minimal depression, 5-9 = mild depression, 10-14 = moderate depression, 15-19 = moderately severe depression and 20-27 = severe depression. Higher the score, sever will be the condition [8-11].

In addition, a questionnaire created on previously deliberated studies was employed. The emotional sphere contains information regarding parents' feelings when they learned about their child's illness and their emotions regarding their caregiving practice. The next domain included questions about understanding of parental sickness, carrier testing, patterns of inheritance, and management. The third domain takes into account social and societal assistance as well as future financial stability in the wake of this tragedy. Their demographic factors were gender, age, marital status, occupation, age at marriage, thalassemia family history, number of affected children, prenatal screening, carrier confirmation prior to marriage and death from thalassemia [9, 11].

2.3. Data Analysis

The data was analysed through descriptive and analytic statistics in SPSS version 20.0. Descriptive statistics were used to determine the means and frequencies of demographic parameters. Difference between the two groups was determined by independent sample t test. Significant determinants were determined by Pearson's correlation.

3. RESULTS

3.1. Sample Characteristics

Two hundred participants were enrolled in this study. Twenty-seven participants (19 from cases and 08 from control) discontinued the questionnaire. They were given the option to resume and only two continued it again while 25 refused to continue. The final number were 175 participants (83 cases and 92 control). The demographic characteristics of 175 members are presented in Table 1. The 91 (52%)

Table 1. Baseline parameters of study participants.

Characteristics of participants	Total	n (%)		P-value
		Cases	Control	
Gender		83	92	
Male	84	40 (48.2)	44 (47.8)	0.599
Female	91	43 (51.8)	48 (52.2)	
Respondents' relationship				
Father	84	40 (48.2)	44 (47.8)	0.599
Mother	91	43 (51.8)	48 (52.2)	
Age (Years)				
15-25	38	21 (25.3)	17 (18.5)	0.002
26-35	85	29 (34.9)	56 (60.9)	
<35	52	33 (39.8)	19 (20.7)	
Average (Years ± SD)		31.4 ± 7.2	31.7 ± 4.2	
Marital status				
Married	137	62 (74.7)	75 (81.5)	0.522
Separated	19	11 (13.3)	08 (08.7)	
Widow	19	10 (12)	09 (09.8)	
Consanguine marriage				
Yes	109	57 (68.7)	54 (58.7)	0.098
No	66	26 (31.3)	38 (41.3)	
Age at marriage				
15-25 Years	93	46 (55.2)	47 (51.1)	0.006
26-35 Years	73	28 (33.7)	45 (48.9)	
<35 Years	9	09 (10.8)	-	
Average (Years ± SD)		25.7 ± 4.8	25.9 ± 2.6	
Employment status				
Businessman	9	03 (3.6)	06 (6.5)	0.08
Employed	55	22 (26.5)	33 (35.9)	
Daily wedges	44	28 (33.7)	16 (17.4)	
Jobless	67	30 (36.1)	37 (40.2)	
Level of education				
Illiterate	31	25 (30.1)	06 (6.5)	<0.000
Under-matric	15	09 (10.8)	06 (6.5)	
Matric	60	22 (26.5)	38 (41.3)	
Intermediate	36	22 (26.5)	14 (15.2)	
≥Graduate	33	05 (06)	28 (30.4)	
Family income				
<8000 PKR	21	11 (13.3)	10 (10.9)	0.185
8000-16000 PKR	98	49 (59)	49 (53.3)	
17000-24000 PKR	51	21 (25.3)	30 (32.6)	
≥25000 PKR	05	02 (02.4)	03 (03.3)	
Heard of thalassemia before				
Yes	50	31 (37.3)	19 (20.7)	<0.000
No	125	52 (62.7)	73 (79.3)	

of the participants (mothers) were female, and their percentage was highest for both studies: cases 43 (51.8%) as well as controls 48 (52.2%). Mean age of the cases and control were 31.4 ± 7.2 and 31.7 ± 4.2 respectively. However, in cases most were above 35 years of age 33(39.8%) and in controls were 26-35 years of age 56 (60.9%).

The majority were married, with 62 (74.7%) in cases and 75 (81.5%) in controls. Consanguine marriages were most likely appearing in both cases 57 (68.7%) as well as controls 54 (58.7%). About 67 (38%) were unemployed, although the majority were women and housewives. Literacy rates were lower in cases than in controls, with 25 illiterates (30.1%) in cases and 6 illiterates (6.5%) in controls. The financial well-being was also compromised in both cases and controls. 21 participants (11 cases and 10 controls) earned less than 8000 PKR per month, whereas just five participants (02 cases and 03 controls) earned more than 25000 PKR per month. As seen in the control group 73 (79.3%), the majority of the population in general is still unaware of thalassemia. More than half of the patients, 52 (62.7%), had not heard of thalassemia prior to their child's diagnosis. At a significance level of 0.05 in cases and controls showed significant associations for age (0.002), age at marriage (0.006), level of education (<0.000), and prior knowledge of thalassemia (<0.000).

3.2. PHQ Scores

PHQ scores that were computed for patients and controls represented in Table 2. At a significance level of 0.05, a significant correlation was found (<0.000). In the control group, none of the subjects scored severely depressed (<14) and number of subjects with moderate depression, 01 (1.1%) was lower as compared to the number in cases 22 (26.5%).

3.3. Feelings Associated with Caregiver Burden

Data represented in Table 3 shows that 31.3% of the 83 cases had a prior thalassemia diagnosis. However, the majority of the parents 59 (71.1%) had only one kid with thalassemia, and the majority were females 47 (56.6%). 17 (20.5%) people indicated that their child died as a result of thalassemia. It was quite distressing that 59 (69.9%) of the parents were uninformed of the nature of thalassemia their kids had. Though, they were all informed of the length of time needed to complete every transfusion. Every two months, about 36 (43.4%) patients required more than three transfusions.

Parents' feelings about the diagnosis and subsequent treatments were also questioned. When asked how they felt when they learned about their child's illness, 31 (37.3%) expressed sadness. However, 16 (19.3%) mentioned stigmatization fear. When asked about experience as caregiver 48 (57.8%) and adjustment to the circumstances 76 (91.06%) most answered positively. Only 32 (38.6%) of the 83 instances studied avoid asking children questions about their treatment effects, duration of existence as well as social life discrimination.

According to the findings of this study, 45 (54.2%) of participants were uninformed about the techniques of diagnosis while 76 (91.6%) did not undertake carrier testing. Nevertheless, 20 (24.1%) of them missed a close carrier testing laboratory and females lacked awareness at the beginning of their diagnosis. Despite this, 41 people who had a thalassaemic child did not seek prenatal diagnosis. However, more than half reported knowing insufficiently 24 (28.9%) or not understanding 20 (24.1%) the disease's process. Additionally, it was discovered that 55 (66.3%) patients were dissatisfied

Table 2. PHQ-score in cases and controls.

PHQ scale	**Total**	**n (%)**	
States of depression		**Cases**	**Control**
Minimal	56 (32)	05 (6)	51 (55.4)
Mild	92 (52.6)	52 (62.7)	40 (43.5)
Moderate	23 (13.1)	22 (26.5)	01 (1.1)
Moderately severe	3 (1.7)	03 (3.6)	-
Severe	1 (0.6)	01 (1.2)	-
Mean score ± SD	-	8.43 ± 3.2	4.61 ± 2.3
P-value	<0.000		

with the medical advice given since it was either inadequate or hard to fully understandable. Positive support was reported by parents from their spouse 53 (63.9%), family 56 (67.5%), society 60 (72.3%) and health personnel 57 (68.7%). Though, 69 (83.1%) of case respondents reported that their financial situation had deteriorated since their child's illness and subsequent treatment. Half of the participants, 42 (50.6%), were more enthusiastic about learning about the course of action and eventual results, while 27 (32.5%) desired to know more about the disease. Univariate analysis results shown in Table 3 revealed that with the exception of fear of stigmatization, spousal support, and thalassemia information, all other characteristics were substantially linked with poor psychosocial state of thalassemic caregivers.

4. DISCUSSION

This study aimed to emphasize the risk and contributing variables for caregivers of thalassemic children. Participant replies underlined the mothers' demands and concerns, which were replicated in prior investigations [9]. Thalassemia impacts not only those children suffering from it but the entire family in the long run [12]. Previous research defined a caregiver as someone who cares for a chronically ill individual for free [11]. Thalassemia is a chronic condition with no long-term treatment, elevates parental concerns regarding puberty delay, body image, frequent absences from school, uncertainty regarding the future which involves career building as well as marriages along with fear of death, making them susceptible to psychological pain at a young age in life. All of this impedes their development into self-sufficient functional adults [2, 12].

The difference in depression severity among nations is influenced by social and environmental factors. Anum and Dastagir [12] previously discovered a connection between the severity of the medical condition and the mother's depression. Depression in caregivers can be associated with their lifestyle. For instance, lack of leisure activities, fewer visits to family and friends, and an unsatisfied mind are all factors that contribute to caretaker depression. Regular transfusions are another factor contributing to the parents' concern and distress. In contrast to earlier studies, participants expressed uncertainty regarding their children's futures, particularly their marriage and careers. They were optimistic about their children's future [13].

Due to the thalassemic child, parents were separated in a few cases 11 (33.3%). Every day presents fresh challenges to a caregiver. Early disease onset and ongoing therapy increase parental pressure at an early age. Aside from disease, the caregiver puts a hardship on other children by devoting time to the affected child. Stress can be enhanced more by enduring anger, regret and humiliation from family members. This issue may be overcome by learning to be patient and generous as well as self-disciplined in the manner to organize their spare time, establish goals, and put forth efforts to achieve them [9].

The problem with our society is that thalassemia-affected children are not accepted. They are stigmatized more frequently, which demoralizes not just the youngster but also rest of the family. Frequent absences from school place the youngster behind in their academics, which is misinterpreted as a lack of cognitive capacities, resulting in a low learner [13]. Furthermore, direct questions from the affected youngster concerning his/her health, profession, marriage, or death operate as a stress stimulant for the caregiver. In some cases, it is preferable to keep the children informed of their health state so that they can take a more active role in their care. In previous investigations, it was found that lower education was linked to worse caregiver outcomes while being female was not a factor [14, 15].

One more serious concern that increased thalassemia's burden was financial concerns. The main cause of an increasing burden on families was an increase in the expenditures of living, transportation, and admittance. Similar results have been obtained in earlier research. Despite the fact that transfusions are free, the cost of transportation and leaving a regular job or place of employment to visit a transfusion clinic can be both mentally exhausting and financially draining [8, 13, 16].

It should be mentioned that such feelings do not occur overnight, but rather as a result of their continual care for their impaired child. This may be caused by a variety of circumstances, including the weakening of family support over time, stigmatization of mothers in particular regarding

Table 3. Psycho-functional capabilities of caregivers (n = 83).

Characteristics	n (%)	OR	*p*-value
Family history			
Yes	26 (31.3)	Ref.	
No	57 (68.7)	4.8 (2.4-9.26)	<0.0001
Number of thalassemic child			
1	59 (71.1)	79.7 (18.3-346.5)	<0.0001
2	22 (26.5)	14.6 (3.3-64.5)	0.0004
3	02 (02.4)	Ref.	
Gender of thalassemic child*			
Male	36 (43.4)	Ref.	
Female	47 (56.6)	1.7 (0.9-3.14)	0.08
Birth order of thalassemic child*			
1	50 (60.2)	29.9 (10-89.5)	<0.0001
2	29 (34.9)	10.6 (3.5-31.9)	<0.0001
3	04 (04.8)	Ref.	
Death of thalassemic child*			
Yes	17 (20.5)	Ref.	
No	66 (79.5)	15 (7-32)	<0.0001
Thalassemia type			
Known	25 (30.1)	Ref.	
Unknown	58 (69.9)	5.4 (2.7-10.4)	<0.0001
Transfusion frequencies			
2/8 weeks	21 (25.3)	Ref.	
3/8 weeks	26 (31.3)	1.3 (0.68-2.65)	0.39
≥ 4/8 weeks	36 (43.4)	2.3 (1.17-4.4)	0.02
Related to Feelings			
Feeling at time of diagnosis			
Shocked	26 (31.3)	3.3 (1.48-7.46)	0.004
Anger	10 (12)	Ref.	
Sorrow	31 (37.3)	4.4 (1.96-9.65)	0.003
Fear of stigmatization	16 (19.3)	1.7 (0.7-4.1)	0.203
Experience faced as caregiver			
Frustrated	35 (42.2)	Ref.	
Non-frustrated	48 (57.8)	1.8 (1-3.5)	0.04
Avoids children question			
Yes	32 (38.6)	Ref.	
No	51 (61.4)	2.5 (1.36-4.75)	0.004
Adjusted with the circumstances			
Yes	76 (91.6)	Ref.	
No	07 (8.4)	117 (39.4-352)	<0.0001
Related to Information			
Carrier testing			
Yes	07 (8.4)	117 (39.4-352)	<0.0001
No	76 (91.6)	Ref.	
Why not consult doctor at time of pregnancy			
Don’t know	45 (54.2)	4.3 (2.17-8.4)	<0.0001
Normal pregnancy	18 (21.7)	Ref.	
No facility available	20 (24.1)	1.15 (0.56-2.4)	0.71
Prenatal screening after thalassemic child			
Yes	19 (22.9)	Ref.	
No	41 (49.4)	3.3 (1.7-6.4)	0.0005
NA	23 (2.7)	1.2 (0.6-2.6)	0.48
Information about thalassemia			
Insufficient	24 (28.9)	1.2 (0.64-2.56)	0.48
Moderately sufficient	12 (14.5)	0.5 (0.24-1.18)	0.12
Don’t understand	27 (32.5)	1.5 (0.77-3)	0.23
Continuous process	20 (24.1)	Ref.	
Guidance from physician			
Insufficient	38 (45.8)	3.3 (1.65-6.5)	0.0007
Moderately sufficient	28 (33.7)	1.98 (0.98-3.98)	0.057
Don’t understand	17 (20.5)	Ref.	

Related to support received				
Support from spouse				
	Yes	53 (63.9)	16.6 (7.04-39)	<0.0001
	Busy with job	08 (09.6)	Ref.	
	End up in sorrow or quarrel	02 (02.4)	0.23 (0.048-1.13)	0.07
	Not alive	20 (24.1)	2.98 (1.23-7.22)	0.02
Support from family				
	Most of the time	56 (67.5)	64.9 (18.6-226.8)	0.3
	Not always available	19 (22.9)	7.9 (2.24-27.9)	<0.0001
	Stigmatization	05 (06)	1.71 (0.39-7.4)	0.473
	Continuous support	03 (03.6)	Ref.	<0.0001
Support from society				
	Yes	60 (72.3)	6.8 (3.4-13.4)	<0.0001
	No	23 (27.7)	Ref.	
Support from health care staff				
	Mostly supportive	57 (68.7)	Ref.	
	Insufficient	06 (07.2)	28.1 (10.86-72.86)	<0.0001
	Biased	08 (09.6)	0.048 (0.02-0.115)	<0.0001
	Didn't notice	03 (03.6)	0.02 (0.005-0.06)	<0.0001
	Sufficient	09 (10.8)	0.06 (0.02-0.128)	<0.0001
Financial status after disease diagnosis				
	Same as before	14 (16.9)	Ref.	
	Lower than before	69 (83.1)	24.3 (10.8-54.7)	<0.0001
Need				
	Emotional control strategy	11 (13.3)	Ref.	
	Information about disease	27 (32.5)	3.16 (1.4-6.9)	0.004
	Time to discuss about treatment and its outcomes	42 (50.6)	6.7 (3.1-14.4)	<0.0001
	Nothing needed	03 (03.6)	0.25 (0.06-0.91)	0.036

OR = odds ratio, Ref. = referent category, values in brackets are range. *P value* ≤ 0.05 was considered significant.

their children's health, and the progression of the illness. In this way family support is insufficient due to excessive demands [17].

It is impossible to overestimate the importance of psychological therapy, as it will have a long-lasting influence on caregiver as well as family along kid [2]. Thalassemia is an illness that requires lifelong care, putting a persistent strain on individuals, their families, and the territory's health organization. Parents may experience unnecessary anxiety and mental discomfort due to the lack of awareness regarding thalassemia and its management [12]. Such details ought to be repeated at regular periods. Doctors and other health care providers can help mothers and their children by incorporating psychological support into their care plans for thalassemic patients [5, 7].

Despite having a positive family history, consanguine marriages are common in a small number of families which contributes to the failure of thalassemia prevention efforts. Due to low literacy rates, parents were not well-informed on the nature of the illness, inheritance patterns, and course of treatment. These issues rise questions that include how mutations happen, why people need transfusions for the rest of their lives, and which medical interventions work best. This knowledge should be reinforced at predetermined intervals, especially while giving medicinal treatments [7, 17]. This requires healthcare professionals particularly doctors to take a role in spreading their message. As a result, educational programs based on parents' educational needs, socioeconomic situation, educational level, gender as well as age are highly recommended. This plan of actions should also be available to the extensive families of present patients. Furthermore, support from a spouse along with family members assisted in lowering the psychological strain linked to thalassemia [2, 18].

To end the misconception that thalassemia is a contagious disease, as well as the acceptance of pre-screening along with premarital exams and unfavourable rejection letters for family members screening due to stigmatisation fears, a physician's responsibility in a community-based awareness campaign must be initiated, in addition to ethical principles and cultural norms. The large number of case individuals with low-income backgrounds may restrict the trustworthiness of our findings. The

poorer psychological condition might be related to the time spent during the transfusion period [19].

5. CONCLUSIONS

Caregivers of thalassemia are exposed to trauma in their very early age that limits their ability to become effectual individuals. Parental anxiety and emotional discomfort might be unintentionally intensified by their lack of understanding of thalassemia and its treatment. As a result, offering comprehensive and appropriate health treatments to these caregivers along with psychiatrist nurses in assisting them to resolve the psychological challenges is valuable. Additionally, promoting caregivers awareness of self-care and manners to care for children with illnesses, establishing periodic educational programs for other family members and close relatives of thalassemia patients, setting up private institutions with the public's involvement despite imposing financial burdens on families, and promoting public awareness about thalassemia through media are all essential. All these strategies must be built considering the parental educational needs, financial status, academic achievement, age as well as gender in order to maintain the health status of caregivers and child with illness.

6. ETHICAL STATEMENT

This study was approved by ethical committee of School of Biological Sciences, the University of the Punjab, Lahore, Pakistan. The mentioned work was done in conformity with the World Medical Association's Code of Ethics (Declaration of Helsinki).

7. CONFLICT OF INTEREST

Authors have no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 217-226 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)911

Pakistan Academy of Sciences

Research Article

Insecticidal Efficacy of Diatomaceous Earth and Botanical Powders against Pulse Beetle, *Callosobruchus analis* F. in Stored Mung Bean (*Vigna radiata* L.)

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Abstract: The pulse beetle is the most damaging insect pest of legumes causing huge economic losses. The experiment was conducted to find out the insecticidal effects of diatomaceous earth (DE) combined with three botanical powders in three different combinations of 25% plant powder with 75% DE, 50% plant powder with 50% DE, and 75% plant powder with 25% DE. The results indicated that the least number of adult beetles settled on grains treated with T_9 (75% GP + 25% DE) and T_3 (75% NP + 25% DE) whereas a maximum number of adult beetles settled in T_4 (25% TP + 75% DE). After 168 hours, all the treatments showed significant mortality (100%) of adult beetles compared to control (43%). All the treatments reduced the number of eggs (3.60 to 6.80 eggs per 20 grains) in comparison to control (17.00). The maximum (28.80) days to adult emergence were documented in T_9 whereas; minimum (21.40) days were recorded in untreated grains. Among all the treatments, T_9: showed minimum emergence (36.40) of adult beetles whereas; the maximum (125.20) adults emerged in control. The lowest grain damage (5.50%) was observed in T_9 as related to the maximum grain damage (28.96%) in the untreated control. The minimum weight loss (3.49%) was recorded in T_9. The maximum weight loss (21.08%) was recorded in untreated grains. The use of diatomaceous earth in combination with garlic powder is recommended for the management of pulse beetle under storage conditions.

Keywords: *C. analis*, Repellency, Insecticidal Efficacy, Biological Effects, Mung Bean.

1. INTRODUCTION

The mung bean (*Vigna radiata* L) is a significant pulse crop among grain legumes grown in Pakistan. It ranks second to Chickpea (*Cicer arietinum*) among the grain legumes in terms of production. Its seeds are very tasty, nourishing, and easily digestible and never cause flatulence than other pulses grown in Pakistan [1]. Approximately one third of the total food production in the world is destroyed by more than 20000 species of pre- and post-harvest insect pests causing more than 100 billion dollars loss annually. The losses caused by insect pests include weight loss, reduced germination potential and reducing the commercial value of the infested grains [2]. The insect pests damage stored grains by consuming grains and contamination by debris of exuviae and cadavers thereby reducing the quality as well as quantity and making the grains unfit for human consumption [3]. The infestation of Bruchids begins in the field before the crop is harvested [4] which is passed to storerooms, leading to higher infestation and damage of stored grains. The suitable environmental conditions including high humidity and temperature facilitates the rapid development of stored grain insect pests. The eggs hatch into larvae which bore into the pulses and start damaging them. Haines [5] reported that *C. analis* is a major pest of the green gram and white soybean.

Losses caused by insect pests both in the field and storage are one of main reasons for pulses production. Approximately 8.5% of total annual production is lost during storage and postharvest handling [6]. Various species of bruchids including *C. chinensis, C. maculates* and *C. analis* have been documented to cause post-harvest damage to stored grain legumes [7]. Among the stored grain pests. the pulse beetle (*Callosobruchus analis* F.) (Coleoptera:

Received: June 2023; Revised: May 2024; Accepted: June 2024
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Bruchidae) is one of the most damaging insect pests infesting mung bean, gram, cowpea, lentil and other pulses and making them unsuitable for human consumption and planting [8, 9]. The larval stage of pulse beetle (*Callosobruchus spp)* inflicts heavy damage as they make holes in grains and consume inner part and cause damage ranging from 32-64%. The damaged grains are unable to sprout and also become inedible for human consumption [10, 11].

Generally, the management of stored grain insect pests is relied on the use of fumigants [12] and also by synthetic insecticides [13, 14], which carry many limitations and ill effects. The indiscriminate use of insecticides may cause serious health hazards as well as the destruction of beneficial insects and lead to increasing costs of application [15-17]. Entomologists throughout the world are seeking efficient, safer, economical and effective methods of pest control in stored grains which can be combined with older practices like sanitation, packaging, better storage facilities and periodical inspection to devise integrated pest management models [18, 19].

Numerous local plant species have been tested in recent years with significant degree of success as protectants against a variety of stored grain insect pests [20, 21]. Insecticidal plants are efficient substitutes for chemical insecticides to trim down pesticide load in the environment [22]. Botanicals have been used in the world for decades to control stored insect pests [23]. Previously, plant aqueous extracts using *Nicotiana tabacum* and *A. indica* caused complete mortality of *C. chinensis* at nine days after treatment of chickpea grains [24].

To substitute the use of synthetic chemicals for nontoxic, natural and effective products against stored grain pests has led to the development of inert dust formulations [25]. Diatomaceous earth is an inert dust, which is derived from amorphous sediments containing fossilized carapaces of unicellular algae. It is a white to dark gray light weight material with low density. It is composed of 80-93% silicon dioxide and the remaining content is comprised of clay minerals, calcium and magnesium carbonate, quartz and organic matter [25, 26]. Shah and Khan [27] reported that diatomaceous earth is a sound option for pest control in grain storage. Diatomaceous earth is abrasive and slightly absorptive, its particles adhere to the insect's cuticle and remove the lipid monolayer, causing the insect to desiccate [25, 28].

Diatomaceous earth has the potential as a protector of grains, because of its safety, efficacy, lower prices and no effect on the final quality of grains [29]. Diatomaceous earths (DEs) are one of the best alternatives to contact insecticides. These dusts can be applied directly to the grain without using any equipment and are nontoxic to mammals [25, 28]. Diatomaceous earths (DEs) can be effectively incorporated into integrated stored grain pest management (ISGPM) program as they possess low mammalian toxicity, and have been found effective against a variety of insect pests [8]. Keeping in view the problems of conventional/ synthetic pesticides, the benefits of botanicals and diatomaceous earths as safe alternative to control pulse beetle and to minimize the losses caused by it during post-harvest storage in Mung bean grains. The present research work was undertaken by using a combination of three indigenous plant powders and diatomaceous earth for the management of pulse beetle.

2. MATERIALS AND METHODS

The experiment was conducted to figure out the insecticidal efficacy of various native plant materials (powders) and diatomaceous earth against pulse beetle (*Callosobruchus annalis* F.) on mung bean in the Post Graduate Laboratory of the Department of Entomology, Faculty of Agriculture, Gomal University, Dera Ismail Khan, Khyber Pakhtunkhwa (KPK), Pakistan.

2.1. Preparation of Insect Culture

Mung bean (*Vigna radiate* L.) seeds, muslin cloth, mesh sieve, plastic jars, funnel etc. were used to prepare the culture of pulse beetle. The infested grains were collected from the local grain market for further multiplication after the identification of *C. analis*. Mung bean grains of locally available variety Dera mung were purchased from the grain market. The seeds were sieved and cleaned to remove the undesired materials. To eliminate the traces of insects, the grains were sterilized. Five hundred grams of disinfested mung bean grains having 12-14% moisture content (mc) in each plastic jar were used to establish the culture of pulse beetle. The moisture content of the seeds was

measured by using OSAW digital moisture meter before starting the experiments. The jars having infested seeds of mung bean enclosed with muslin fabric were retained in an incubator (Versatile Environment test Chamber, Sanyo Japan, Model-MLR-350 H) at a controlled temperature of 27 ± 3 °C, 65 ± 5% R.H. and a photoperiod of 12:12 hours (Light: Dark) till the emergence of adult beetles following standard method described by Zafar *et al*. [30]. Healthy adults emerged from the container were shifted to the other plastic jars (having 500-gram mung bean seeds) for oviposition purpose. The mouths of the jars were closed with muslin fabric and tightened with elastic bands to avoid escape of pulse beetle and to allow air in and out of the jars. After 168 hours, the pulse beetles were removed from the jars with the help of mesh sieve and introduced into other jars to multiply the insect culture. The jars having infested mung bean seeds were retained undisturbed for ten days. The newly emerged subsequent generations of pulse beetle were utilized for the investigations.

2.2. Plant Materials and Diatomaceous Earth

The plant materials including neem, *Azadirachta indica* (seeds), turmeric, *Curcuma longa* (Rhizomes) and Garlic, *Allium sativam* (bulbs) were obtained from the field, local market as well as local growers. The plant materials were shade dried and used to prepare powders for experiments and maintained under constant environmental conditions until used. Diatomaceous earth imported from China was utilized in combination with various plant powders.

2.3. Preparation of Plant Powders

The plant materials used in the study were dried in shade. The dried plant materials were milled separately into very fine powder with the help of an electric grinder and sieved using 2mm mesh sieve to get fine powders and stored in air tight bottles.

2.4. Experimental Procedure

2.4.1. Settling response

An experiment was conducted to investigate the impact of the aforementioned native plant materials (powders) and diatomaceous earth on the settling response of pulse beetle on mung bean. The settling response of the *C. analis* was investigated using 30 × 30 cm^2 circular plastic boxes. For the investigations, there were ten treatments having five repeats. For the experiment, newly emerged adult beetles were collected. The beetles were kept starved for an hour before the start of the trial. A group of 50 newly emerged adult beetles were selected and were released in the middle of the plastic boxes to find out the effect of selected treatments on the settling response of beetles. The data was recorded after 24, 48 and 72 h of the release of the adult beetles and was converted to a percent settling response. While recording the data on the settling response of adult beetles, those beetles that did not respond, i.e., they did not settle on the treated grains and settled on different parts of the arena or remained unmoved were also recorded.

2.4.2. Insecticidal and biological effects

The experiment was conducted to study the insecticidal effects of diatomaceous earth (DE) combined with three selected botanical powders. The plant powders were mixed with the diatomaceous earth in three different combinations consisting of 25% Plant powder with 75% DE, 50% Plant powder with 50% DE and 75% Plant powder with 25% DE. All the treatments were used at 500 PPM. The studies were carried out in an incubator set at 27 ± 3°C and 65 ± 5% relative humidity. There were ten treatments having five replications. In every treatment, 50 grams of sanitized mung bean seeds were kept in transparent plastic jars which were thoroughly treated with a combination of DE and Plant powders. Jars for each dose were kept undisturbed for 30 minutes to allow the dust particles to settle down. The diatomaceous earth concentrations were prepared by following the standard procedure described by Fields *et al*. [31]. After the treatment of mung bean grains five pairs of newly emerged pulse beetle adults for each treatment were introduced in each jar and then the jars were closed tightly with cotton cloth to inhibit the movement of beetles. At 168 hours after the treatment of mung bean grains, the parent insects were removed and the number of eggs laid on 20 grains were noted and the mean was calculated. The jars were checked daily for recording data on the number of days to adult appearance. The number of adults emerged were counted daily and continued up to 40 days and later on were added to determine the total number of adult pulse beetles that emerged and then the average was calculated.

Mortality data was recorded after 24, 48, 72 and 168 hours, respectively, of the release of beetles. The dead adult beetles were collected and removed after each observation. The sublethal effects of the selected treatments were confirmed by recording data on the number of eggs per 20 grains, days to F_1 adult emergence, overall emergence of adult progeny, infestation (%), percentage weight loss, sex percentage (male and female ratio) and life span of adult beetles. The percent mortality was calculated by following formula as used by Ahmed *et al*. [32] and weight loss of grains were calculated by following formula as used by Salim *et al*. [33].

$$\% \, Mortality = \frac{No.of\ dead\ weevils}{Total\ No.of\ weevils\ released} \times 100$$

$$\% \, weight\ loss = \frac{weight\ of\ control\ grains - weight\ of\ treated\ grains}{weight\ of\ control\ grains} \times 100$$

2.5. Statistical Analysis

The data recorded were statistically analyzed using computer software Statistix Ver. 8.1 at $P < 0.05$ using one-way analysis of variance technique.

3. RESULTS

3.1. Settling Response

The data indicated in Fig. 1 show varying degree of repellent effect of selected treatments against pulse beetle on mung bean. The data regarding the settling response of pulse beetle show that after 24 hours the least number of adult pulse beetle settled on the mung bean grains treated with T_9 (75% GP + 25% DE), T_3 (75% NP + 25% DE) and T_6 (75% TP + 25% DE), whereas; maximum number of adult beetles settled on T_4 consisting of 25% TP + 75% DE, T_1 consisting of 25% NP + 75% DE and T_7 consisting of 25% GP + 75% DE, respectively. At 48 and 72 hours after the treatment an almost similar settling response of pulse beetle was noted (Fig. 1). Overall; T_3 and T_9 were found most-effective treatments resulting in minimum settling response whereas; T_4, T_1 and T_7 were found least effective treatments having minimum effect on the settling response of adult pulse beetle.

3.2. Mortality of Pulse Beetle after 24 Hours

Mortality of adult pulse beetle recorded after 24 hours of the application of diatomaceous earth and plant powders was found significantly different ($P < 0.05$) (Table 1) in all the treatments as compared to mortality (lowest) observed in the control. However, among all the nine treatments 75% GP (Garlic powder) + 25% DE and 75% TP + 25% DE showed the maximum mean mortality (27.80% and 26% respectively) of adult pulse beetle having significant variation from the rest of the treatments. The effectiveness of the treatments after turmeric powder was followed by 50% GP (Garlic powder) + 50% DE (25.20%) and 50% TP + 50% DE (24.60%). The minimum mortality of adult beetles was recorded in 25% TP (Turmeric powder) + 75% DE (22%). No mortality of the pulse beetle was recorded in the untreated control.

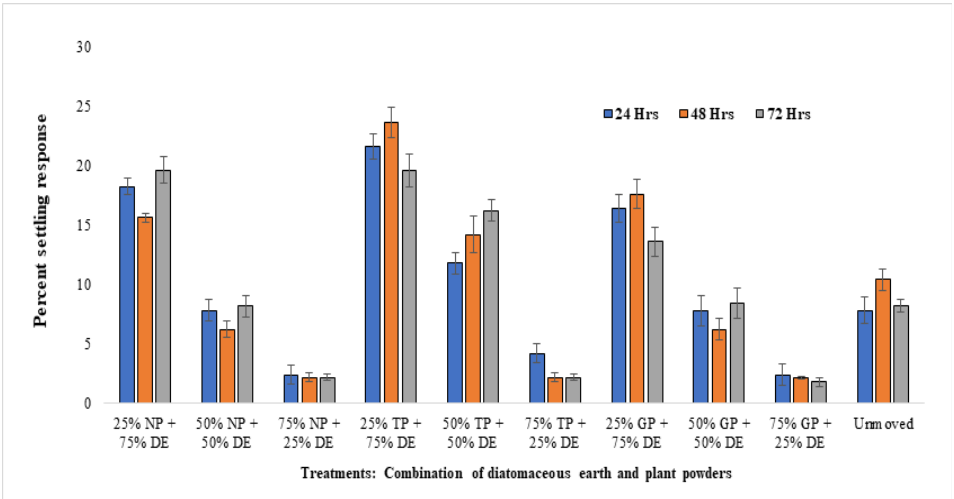

Fig. 1. Impact of plant powders and diatomaceous earth on the settling response of *C. analis* on mung bean grains under laboratory conditions.

3.3. Cumulative Mortality of Pulse Beetle after 48 Hours

Cumulative mortality of adult pulse beetle noted after 48 hours of the application of three plant powders in combination with diatomaceous earth (DE) was found significantly different ($P < 0.05$) in all treatments compared to the control. However, among all the treatments 75% GP (garlic powder) + 25% DE showed maximum mean mortality (69.60%) of pulse beetle adults being statistically different from all other treatments. It was followed by 50% GP + 50% DE and 75% TP + 25% DE showing 61.80 and 61% mortality of adult beetles having no significant variation from each other (Table:1). All the remaining six treatments showed significant mortality of (47.80% to 50.60%) of adult beetles having no significant variation from each other. The minimum mortality (2.40%) of adult pulse beetle was recorded in the control treatments (Table 1).

3.4. Cumulative Mortality of Pulse Beetle after 72 Hours

Collective mortality of pulse beetle recorded after 72 hours of the application of treatments was found significantly different ($P < 0.05$) in all treatments as compared to control where minimum mortality was observed (Table 1). However, among all the treatments, T_9 (75% garlic powder + 25% diatomaceous earth) caused the maximum mortality having non-significant difference from T_8, T_7, T_6 and T_5, having significant variation from the rest of the treatments. The control treatments were found least effective having minimum mortality (5.40%) of pulse beetle. All the treatments after 72 hours were found very effective in controlling the pulse beetle as compared to untreated control.

3.5. Collective Mortality of Pulse Beetle after 168 Hours

Combined mortality of pulse beetle noted after seven days of the application of three plant powders in combination with diatomaceous earth (DE) was found significantly different ($P < 0.05$) in all the treatments as compared to control where minimum mortality was recorded. All the treatments showed highest mortality (100%) of pulse beetle adults being statistically similar with each other having significant variation from untreated control having minimum mortality (13.80%) of pulse beetle at seven days after the exposure period (Table 1). It was observed that the rate of mortality increased with the increase of exposure period at all concentrations/ combinations of plant powders and DE.

3.6. Number of Eggs per 20 Grains

The recorded data revealed that a significantly higher number of eggs were observed in untreated grains compared to plant powders plus diatomaceous earth treated mung bean grains ($P < 0.05$). All the combinations of botanical powders and diatomaceous earth significantly inhibited oviposition by pulse beetle. The minimum number of eggs (3.60) were noted on mung bean grains treated with 75% GP + 25% DE having significant variation from all other treatments. It was followed by 50% GP + 50% DE recording 4.40 eggs. Overall,

Table 1. Percent mortality of pulse beetle at different concentrations of plant powders and diatomaceous earth after 24 h, 48 h, 72 hours and 168 hours exposure period.

Treatments	24-hours	48-hours	72-hours	7-days
25% NP + 75% DE	22.80 ± 1.11 cd	48.00 ± 0.43 c	91.60 ± 3.17 d	100.00 a
50% NP + 50% DE	23.00 ± 1.23 bcd	49.80 ± 1.27 c	92.80 ± 1.34 cd	100.00 a
75% NP + 25% DE	24.20 ± 0.45 bcd	50.60 ± 1.39 c	95.40 ± 1.34 bc	100.00 a
25% TP + 75% DE	22.00 ± 0.76 d	47.80 ± 1.11 c	92.20 ±1.37 cd	100.00 a
50% TP + 50% DE	24.60 ± 1.29 bcd	49.20 ± 1.26 c	97.20 ± 2.11 ab	100.00 a
75% TP + 25% DE	26.00 ± 2.11 ab	61.00 ± 2.27 b	98.80 ± 1.29 a	100.00 a
25% GP + 75% DE	23.00 ± 1.76 bcd	49.40 ± 2.34 c	96.40 ± 2.16 ab	100.00 a
50% GP + 50% DE	25.20 ± 1.19 abc	61.80 ± 3.56 b	98.80 ± 2.33 a	100.00 a
75% GP + 25% DE	27.80 ± 1.32 a	69.60 ± 2.45 a	99.40 ± 1.67 a	100.00 a
Control	0.00e	2.40 ± 1.11 d	5.40 ± 1.15 e	13.80 ± 1.45 b
$LSD_{0.05}$	3.11	3.51	3.25	0.99

Mean values having different letter(s) are significant at $P < 0.05$

the maximum number of eggs (17.00 eggs per 20 grains) were recorded in control treatments (Table 2).

3.7. Days to F_1 Adult Emergence

The application of three plant powders combined with diatomaceous earth had a significant effect ($P < 0.05$) on the number of days to adult emergence of pulse beetle compared to the untreated control. The maximum (28.80) days to adult emergence were documented in T_9 having non-significant variation from 50% GP + 50% DE, 75% NP + 25% DE, 75% TP + 25% DE, 25% GP + 75% DE and 50% TP + 50% DE. The minimum duration to adult emergence was recorded in untreated grains (21.40 days) being statistically different from all the other tested treatments (Table 2).

3.8. Total F_1 Adult Emergence

Collective emergence of F_1 adult beetles from mung bean grains recorded after the application of three plant powders in combination with diatomaceous earth (DE) was found significantly different ($P < 0.05$) in all treatments compared to control where maximum adult emergence was noted (Table 2). However, among all the treatments T_9: 75% GP (garlic powder) plus 25% DE (diatomaceous earth) showed minimum emergence of adult beetles (36.40 mean) having non-significant difference from T_8: 50% GP + 50% DE, T_7: 25% GP + 75% DE, T_6: 75% TP (turmeric powder) plus 25% DE (40.60, 41.20 and 44.80 mean respectively). The maximum mean number of adults emerged (125.20 mean) were noted from untreated mung bean grains.

3.9. Percent Infestation of Mung Bean Grains

At 40 days after the treatment of mung bean grains, the infested mung bean grains in different treatments were found significantly less as compared to untreated control (Table 3). The data revealed that all the treatments performed better in reducing the damage of grains compared to untreated control that sustained significant ($P < 0.05$) seed damage due to infestation and feeding by *C. analis*. The maximum grain damage (28.96%) was observed in untreated control and the lowest grain damage ranging from 5.50 to 9.09% was recorded in all the treatments of plant powders plus diatomaceous earth being significant to untreated control. Among the treatments, the lowest grain damage (5.50%) was observed in T_9 consisting of 75% GP (garlic powder) + 25% DE followed by T_6: 75% TP + 25% DE and T_7: 25% GP + 75% DE (6.42% and 6.59% respectively) being statistically at par with each other. All the remaining treatments were also effective in reducing the percent grain damage as compared to control.

3.10. Weight Loss of Mung Bean Grains

The use of botanical powders in combination with diatomaceous earth significantly reduced the weight loss of mung bean grains caused by *C. analis* compared to untreated control (Table 3). Among the treatments, the minimum weight loss of 3.49% was recorded in T_9: 75% GP (garlic powder) + 25% DE having non-significant variation from T_8: 50% GP + 50% DE, T_6: 75% TP + 25% DE and T_7: 25% GP + 75% DE. However, all the treatments

Table 2. Number of eggs per 20 grains, days to F_1 adult emergence and Total F_1 adult emergence of pulse beetle on mung bean grains.

Treatments	No. of eggs/20 grains	No. of days to F_1 adult emergence	Total F_1 adult emergence
25% NP + 75% DE	6.80 ± 0.12 b	24.80 ± 1.12 b	61.00 ± 1.23 b
50% NP + 50% DE	6.40 ± 0.15 bc	25.20 ± 1.13 b	51.20 ± 0.36 cd
75% NP + 25% DE	5.40 ± 0. 17 bc	28.00 ± 0.77 ab	51.00 ± 0.19 cd
25% TP + 75% DE	6.20 ± 0.19 bc	25.00 ± 0.47 b	55.20 ±1.34 bc
50% TP + 50% DE	5.60 ± 0.11 bc	25.80 ± 0.39 ab	51.60 ± 1.23 cd
75% TP + 25% DE	5.80 ± 0.22 bc	27.80 ± 0. 44 ab	44.80 ± 1.45 de
25% GP + 75% DE	5.20 ± 0. 17 bc	27.00 ± 0.39 ab	40.60 ± 1.29 ef
50% GP + 50% DE	4.40 ± 0.19 bc	28.00 ± 1.23 ab	41.20 ± 2.16 ef
75% GP + 25% DE	3.60 ± 0.11 c	28.80 ± 1. 37 a	36.40 ± 1.39 f
Control	17.00 ± 1.11 a	21.40 ± 1.23 c	138.20 ± 3.23 a
$LSD_{0.05}$	2.98	3.30	8.25

Mean values having different letter(s) are significant at $P < 0.05$

were found significantly effective in reducing the percent weight loss of grains compared to untreated control where maximum weight loss (21.08%) of mung bean grains was recorded.

3.11. Sex Ratio

The sex ratio of adult *C. analis* did not differ significantly ($P < 0.05$). The mean sex ratios (44.60 to 45.80% males) of emerged adult pulse beetles showed that the number of males were less in all the treatments as compared to female beetles. However, non-significant results were observed among all the treatments of plant powders plus diatomaceous earth and control (Table 3).

3.12. Adult Longevity

The adult longevity of newly emerged beetles in all the treatments was found statistically significant compared to the control ($P < 0.05$). All the plant powders plus diatomaceous earth treatments had a significant effect on the life span (6.80 to 9.40 days) of adult pulse beetle (Table 3). The minimum adult life span of 6.80 days was recorded in T_9 (6.80%) having non-significant variation from T_3, T_6, T_4, T_5, T_8 and T_7. The maximum adult longevity of 12.00 days was recorded in T_{10} (control).

4. DISCUSSION

There have been certain studies on the efficacy of plant powders and diatomaceous earth (DE) against stored grain pests. However, there is a dearth of available information on the usage of plant powders and diatomaceous earth (DE) in combined form against *C. analis*. The results of our research showed that binary mixes of botanical powders and diatomaceous earth have insecticidal potential against *C. analis*. All the treatments caused high mortality of adult beetles from 24 to 96 hours after treatment. However, adult mortality rapidly increased after 48 hours contact period. Similarly, all the tested treatments had significant effect on the settling response of pulse beetle. It is evident that among the tested treatments, the least number of adult pulse beetle settled on the mung bean grains treated with 75% GP + 25% DE, 75% NP + 25% DE and 75% TP + 25% DE, whereas, the maximum number of adult beetles settled on untreated mung bean grains.

The reason for the better efficacy of plant powders plus diatomaceous earth (DE) could be ascribed to the existence of various constituents in plant powders and DE that synergized with each other and enhanced the efficiency of applied mixtures. The findings of our research are similar to former research work which revealed that stored grain insect pests including *C. analis* can be managed by using plant powders in combination with diatomaceous earth. Paponja *et al*. [34] tested diatomaceous earth (DE Silico Sec) enhanced with plant products (bay leaves dust, corn oil and essential oil lavender) and silica gel against *Tribolium castaneum, Sitophilus oryzae* and *Rhyzopertha dominica*) in stored barley and wheat. Results indicated that botanicals enhanced the activity of diatomaceous earth, SilicoSec and showed great mortality and inhibited the emergence of new progenies of tested insect pest species. Similarly, Athanassiou *et al*. [35] tested the insecticidal

Table 3. Percent infestation, percent weight loss, sex ratio and life span of adult pulse beetle on mung bean grains.

Treatments	Percent infestation	Percent weight loss	Sex ratio $^{N.S.}$	Life span of adult beetles
25% NP + 75% DE	9.09 ± 0.21 b	6.12 ± 0.13 b	45.80 ± 1.44	9.40 ± 0.28 b
50% NP + 50% DE	8.92 ± 0.32 b	5.49 ± 0.07 bc	44.80 ± 1.65	9.00 ± 0.38 b
75% NP + 25% DE	7.24 ± 0.11 bcd	4.89 ± 0.11 bcde	45.00 ± 2.11	7.40 ± 0.31 bc
25% TP + 75% DE	8.52 ± 0.36 bc	5.13 ± o.13 bcd	44.80 ± 1.54	8.80 ± 0.11 bc
50% TP + 50% DE	7.37 ± 0.12 bcd	4.69 ± 0.18 bcde	45.00 ± 1.87	8.80 ± 0.13 bc
75% TP + 25% DE	6.42 ± 0.38 cd	4.11 ± 0.09 cde	44.80 ± 1.76	8.00 ± 0.15 bc
25% GP + 75% DE	6.59 ± 0.33 cd	4.39 ± 0.13 cde	45.00 ± 1.23	8.80 ± 0.14 bc
50% GP + 50% DE	6.95 ± 0.17 d	3.86 ± 0.11 de	45.20 ± 1.76	8.60 ± 0.17 bc
75% GP + 25% DE	5.50 ± 0.19 d	3.49 ± 0.16 e	44.60 ± 1.77	6.80 ± 0.41 c
Control	38.96 ± 1.23 a	27.08 ± 1.33 a	45.60 ± 2.22	12.00 ± 0.23 a
$LSD_{0.05}$	2.15	1.49	1.78	2.08

Mean values having different letter(s) are significant at $P < 0.05$

activity of bitter barkomycin (BBM) prepared from the roots of the *Celastrus angulatus*, alone or as a mixture with diatomaceous earth against adults of the rusty grain beetle, *Cryptolestes ferrugineus*, *S. zeamais* and *T. castaneum*. A combination of BBM and DE at all the tested concentrations was found very effective in reducing the infestation of all three stored grain insect pests within 14 days and in decreasing the progeny production of the tested insect pests. These results are in conformity with the previous research findings [36-38].

Plant powders in combination with diatomaceous earth (DE) significantly reduced F_1 adult emergence in all the treatments compared to untreated control. The suppression of F_1 adult emergence may be due to less oviposition or toxic effects of treatments on eggs laid. This is in accordance with Paponja *et al*. [34] who reported a significant reduction of *C. analis* adults in mung bean grains treated with plant powders combined with diatomaceous earth (DE). The present research also corroborates with the findings of Ofuya *et al*. [39], they reported that the mixtures of *Piper guinese* seed powder and diatomaceous earth (DE) significantly reduced the oviposition and F_1 adult emergence of pulse beetle on stored cowpea. From our findings it was observed that grains treated with plant powders and diatomaceous earth (DE) showed reduced F_1 adult emergence, however, complete suppression of F_1 adult emergence was not recorded, which may be due to the potential of female adult beetles to lay eggs in unfavorable conditions. This is in agreement with Athanassiou [40], who stated that progenies of rice weevil, emerged even in those treatments of stored wheat which were treated with a combination of betacyfluthrin and *diatomaceous earth* (DE) where 100% mortality of adult rice weevils was recorded. The life duration of newly emerged adult pulse beetles in all the treatments were found statistically significant compared to control. All the plant powders plus diatomaceous earth (DE) treatments had a significant effect on the life span (6.80 to 9.40 days) of adult pulse beetle. Our results are similar to Yankanchi and Lendi [41]. They tested various plant powders against pulse beetle and found them effective in reducing the longevity and oviposition by *C. chinensis*.

5. CONCLUSIONS

The combined use of diatomaceous earth along with garlic and turmeric powders possessed repellent and insecticidal properties against pulse beetle in stored mung bean. The minimum number of beetles settled on treated grains. The maximum mortality and minimum progeny emergence of the beetle adults was documented on mung bean grains treated with the aforementioned products. Based on the results it can be concluded that combination of diatomaceous earth and plant powders (garlic and turmeric) are highly effective for the management of pulse beetle (*C. analis*) in stored mung bean. However, further investigations are needed to assess the use of garlic and turmeric powders under field and conventional storage conditions. The use of DE in combination with garlic and turmeric powders would be a good alternative to synthetic chemicals. This study will help to the development of natural pesticides as a vital part of pest management strategies.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 227-234 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)849

Pakistan Academy of Sciences

Research Article

Assessment of Hepatic Enzyme Derangements in Patients with Covid-19

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Abstract: About half of the patients with Covid-19 have deranged hepatic enzymes at presentation. The goal of the study was to identify specific patterns of abnormalities in enzymes so that clinical treatment and therapeutic methods can be improved for affected individuals. This understanding is crucial for improving patient outcomes and creating individualized treatment schedules. A total of 182 RT-PCR-confirmed Covid-19 cases were enrolled and different biochemical variables were compared among patients with varying degrees of disease severity. Data with abnormal distribution were described as median (minimum-maximum) and analyzed with the Mann-Whitney U test and the Kruskal-Wallis test. Multivariate binary regression analysis was applied to find the predictors associated with disease severity. The mean age of patients was 56.46 ± 15.60 years. Median AST levels in 182 patients were more than ALT at admission (52.45 vs. 46.35 U/L. Most of the subjects with the deranged hepatic enzyme at presentation had minimal elevations 1-2X upper limit of normal (ALT 74.8%, AST 77.0%, TBIL 98.3%). An increase of ≥ 5 times the upper limit of normal was observed in 7 (3.8%) and 5 (2.7%) patients for AST and ALT, respectively. Non-survivors were older, had higher median levels of AST 70 U/L vs. 47 U/L, LDH 855 vs 470 (for both p-value = 0.0001), and had a longer hospital stay compared to discharged groups. In multivariate analysis, advanced age, raised level of LDH and extended hospital stay showed a significant association with mortality. Liver dysfunction is commonly observed in hospitalized subjects and may be linked to severe disease.

Keywords: Alanine Aminotransferase, Covid-19, Total Bilirubin, Liver Damage, Aspartate Aminotransferase.

1. INTRODUCTION

Since its eruption from Wuhan, China, Coronavirus disease (Covid-19) is linked with considerable morbidity and mortality [1]. Covid-19 exhibits a wide range of clinical presentations extending from lack of overt signs or only mild symptoms in 81% to critical disease in 5% of the patients [2]. Although primarily manifested as pulmonary infection, liver impairment is a highly reported complication of Covid-19. Around 14–76% of Covid-19 patients had raised concentrations of hepatic enzymes, i.e., alanine aminotransferase (ALT) or aspartate aminotransferase (AST) [3] Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the causative pathogen in Covid-19. It shares about 79.6% sequence with SARS-CoV-1targets the Angiotensin-Converting Enzyme 2 (ACE2) receptor to enter the body of the host. ACE2 is expressed in various cells, including hepatocytes, cholangiocytes in the biliary epithelium, and alveolar cells in the lungs. In the lungs, the virus binds to ACE2 on alveolar cells, leading to inflammation and respiratory complications, which can result in conditions like pneumonia and acute respiratory distress syndrome (ARDS) [4]. In recent times, 60% of SARS patients had extensive liver damage [5]. SARS-CoV-2 may likewise have hepatotoxic effects because it belongs to the same family and can attach directly to ACE-2 receptors found on hepatocytes and cholangiocytes. Due to the lack of prior immunity and the incomprehensible nature of the disease, clinical assessment, and patient management protocols are

Received: February 2023; Revised: May 2024; Accepted: June 2024
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perplexing yet evolving. Deranged liver enzymes are prominently identified as an extra-pulmonary clinical manifestation of Covid-19 infection reported by at least one-third of the Covid-19 infected subjects. This research was done to determine how individuals with Covid-19 were affected by hepatic dysfunction in terms of disease severity and prognosis.

2. METHODOLOGY

This cross-sectional observational study was carried out at the Department of Pathology, Benazir Bhutto Hospital, Rawalpindi, from June 8th, 2020 to December 30th, 2020. One hundred and eighty-two, RT-PCR-confirmed Indoor Covid-19 patients were enrolled by consecutive sampling. Patients were followed up to the final clinical outcome. Liver Function Tests (LFTs), including, Total Bilirubin (TB), Aspartate Aminotransferase (AST) Alanine Aminotransferase (ALT), and Lactate Dehydrogenase (LDH) were assayed on an Automatic Chemistry Analyzer Beckman Coulter Au - 480 at presentation, and peak values during hospital stay were noted. Subjects with a known history of hepatic dysfunction were not enrolled in the study. Liver dysfunction and reference ranges for increased aminotransferases were defined according to the guidelines of the European Association for the Study of the Liver [6]. Baseline data inclusive of gender, age, and duration of hospital stay were collected. Subjects were grouped into mild, moderate, and severe according to Clinical Management Guidelines for COVID-19 Infections v3 Document Code: 12-03 Date: July 2nd, 2020, Pakistan [7]. The patient's age, sex, hepatic enzyme, ALT, AST, TB, LDH, and ferritin were assessed at admission. All the variables were compared among patients with varying degrees of disease severity (Figure 1).

2.1. Statistical Analyses

Normally distributed parameters were presented as means ± SD and analyzed with an independent sample t-test and One-way ANOVA. Data with abnormal distribution were analyzed with the nonparametric tests and nominal data were analyzed with the Chi-square test. Multivariate regression was used to find the association between hepatic dysfunction, disease severity, and mortality. Mild or moderate/severe were dependent variables, and response variables included: ALT, AST, TB, LDH, ferritin, and hospital stay. The odds ratio (OR) with a 95% confidence interval (CI) was measured for each variable. Graph pad prism and SPSS version 25 were used to analyze the data. A p-value < 0.05 was considered significant.

3. RESULTS

The mean age of the participants was 56.46 ± 15.60 years (17-92). The majority of the patients, 119 (65.4%), were male, and 77(42.3%) were more than 60 years of age. The median hospital stay for severe patients was ten days, and a predominantly high percentage of severe patients (84.2%) succumbed to death compared with the subjects with a mild and moderate degree of 'disease' (Table1).

3.1. Distribution of Laboratory Parameters at Presentation

Median concentrations of AST, ALT, TB, LDH, and ferritin were significantly higher in the severely infected group compared to the mildly and

Table 1. Comparison of study variables according to disease severity.

Admission characteristics	Data (n = 182)	Mild (n = 47)	Moderate (n = 78)	Severe (n = 57)	p-value
Age (years)≠	59 (17-92)	46.53±16.82	55.49±12.64	65.96±12.58	0.0001
Sex (male)	119 (65.6%)	30 (25.2%)	49 (41.2%)	40 (33.6%)	0.501
ALT U/L*	47 (12-1209)	27.0 (12-70)	54 (14-157)	71 (12-1209)	0.0001
AST U/L*	53 (16.5-724.7)	33 (17-109)	54 (24-131)	88 (31-630)	0.0001
TB mg /dl*	0.45 (0.3-8.0)	0.5 (0.3-1.40)	0.6 (0.3-2.3)	0.6 (0.3-8.0)	0.025
LDH *	484 (170-3922)	429 (169-619)	498 (220-936)	856 (344-3922)	0.0001
Ferritin*	440 (15-1000)	126 (15-620)	399 (31-1000)	738 (46-1000)	0.001
Hospital stay*	5 (1-46)	1 (1-24)	6 (1-28)	11 (1-46)	0.0001
Mortality	66 (36.3%)	5 (10.6%)	13 (16.7%)	48 (84.2%)	0.0001

*Kruskal Wallis test ≠ One Way ANOVA

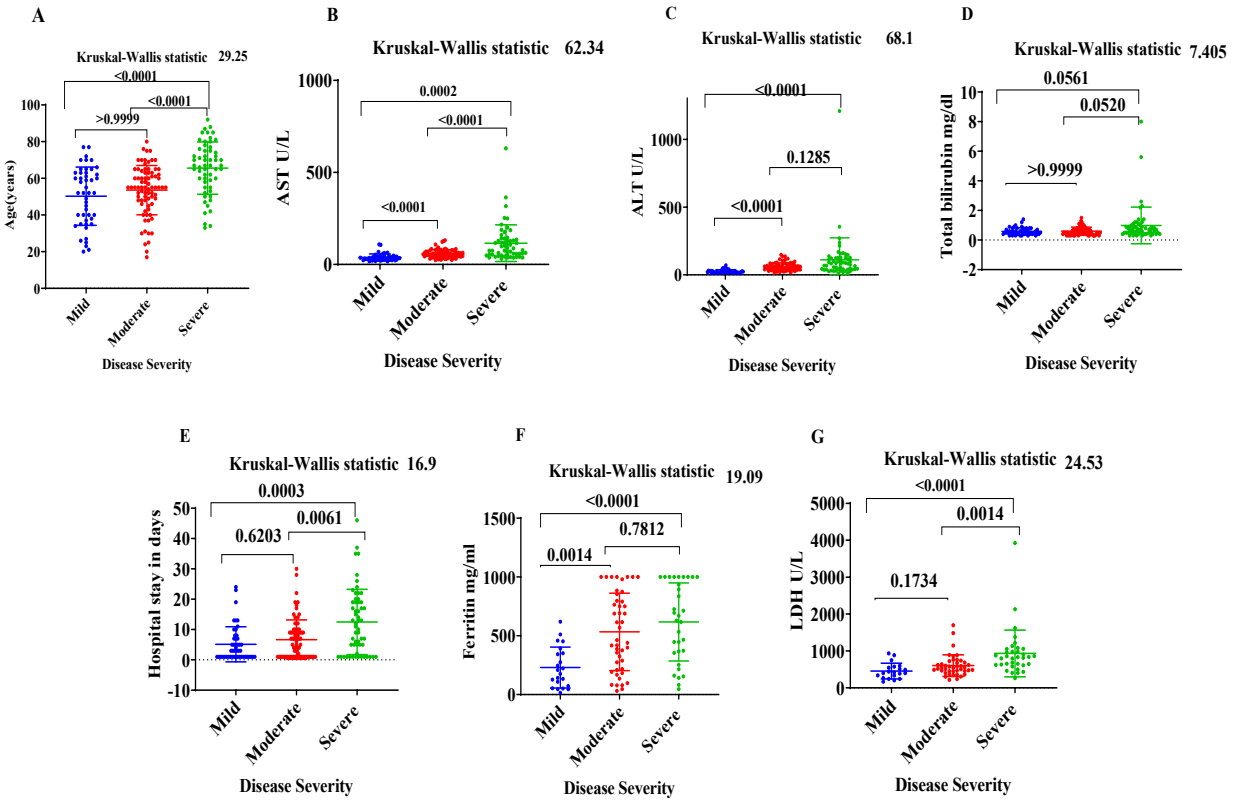

Fig. 1. Post hoc comparisons of A (age), B (Aspartate transaminase), C (Alanine transaminase), D (Total bilirubin), E (Hospital stay), F (Ferritin), G (Lactate dehydrogenase) with disease severity.

moderately affected group. At admission, median AST concentrations were higher than ALT (58 vs. 47 U/L). Covid-19 patients were classified into different groups based on the liver transaminase level. A higher number of patients have raised levels (≥ 40) of AST 126 (69.2%) compared to elevations in ALT 104 (57.1%). Elevations in total bilirubin were rare. Only 3 (1.5%) have ≥ 2 upper limits of normal (ULN) bilirubin. In the majority of patients, elevations in hepatic enzymes were up to 1-2 ULN, while an increase of more than five times ULN was observed in 7 (3.8%) and 5 (2.7%) patients for AST and ALT, respectively (Appendix 1). In comparison to subjects with normal aminotransferases, patients with elevated transaminase activity were significantly older, had elevated ferritin (p-value = 0.001), and LDH concentrations (p-value = 0.003), as well as a longer hospital stay (p-value = 0.032). No significant change in bilirubin levels was seen between the two groups (Appendix 2). Out of 57 critically ill patients, 43 (75.4%) succumbed to death, and 14 survived. Nonsurvivors were older (67 vs. 50 years; p = 0.0001) and had longer hospital stays (4 vs. 9 days; p = 0.001) compared to the survivors. Additionally, non-survivors had significantly elevated levels of AST median (min-max) (69.7 vs 46.9 U/l, p = 0.0001); LDH (870 vs 470 U/l, p = 0.0001) and bilirubin (0.5 vs 0.7, p = 0.038) (Appendix 3).

3.2. Correlation of AST with LDH and Ferritin

The relationship between AST and other markers of disease severity was evaluated. AST showed a highly significant correlation with ALT and LDH throughout the hospital stay (r = 0.731), (r = 0.503), (p-value = 0.0001), whereas the correlation between AST and ferritin was moderate (Figure 2).

3.3. Univariate and Multivariate Regression Analysis

In multivariate regression analysis, age, LDH, and longer hospital stay showed a significant association with disease severity (Table 2). AST is a nonspecific marker of liver damage. When the multivariate regression model was further adjusted for LDH, AST lost its significance indicating the multi-organ source of AST.

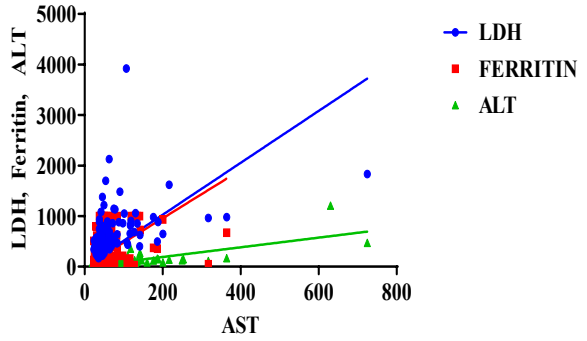

Fig. 2. Correlation of AST with ALT, LDH, and ferritin.

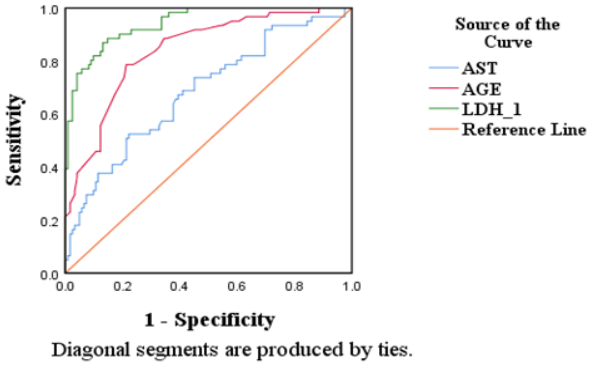

Fig. 3. ROC curve analysis for Age, AST, and LDH.

3.4. Receiver Operator Curve (ROC) Analysis

ROC curves were performed for the variables that showed a significant association with disease severity in multivariate regression analysis. LDH, with a cutoff value of 615 U/l, was the most significant predictor (specificity of 80.0% and sensitivity of 83.0%). Age with a cutoff value of 59.5 years had a high sensitivity (75.6%) and specificity (71.7%), and AST with a cutoff value of 59 U/l (sensitivity 60.0% and specificity 61.30%) remained significant as shown in Fig (3) and Table (3).

4. DISCUSSION

Recently, several international studies reported some degree of liver damage, mainly manifested as the raised concentration of aminotransferases in patients with Covid-19. Our results concur with recent literature, which claims that severely infected subjects are more likely to develop elevated transaminases than mild or moderately infected patients [3, 7-9]. In the current study, high levels > 40 U/L of AST and ALT have been observed in 69.4% and 57.4% of patients with Covid-19. This percentage is higher than observed in a study conducted on 1099 Chinese patients, where raised concentrations (> 40 U/L) of AST and ALT were found in 39.4% and 28.1% of the subject respectively [10]. Similarly, in a study including 554 Turkish participants, elevated transaminases at presentation were observed in 27.6% of the patients [11]. In an American study conducted on 5700 patients, AST and ALT levels

Table 2. Univariate and multivariate regression analysis.

Univariate regression analysis			**Model 1**		**Model 2**	
Variables	OR (95%CI)	p-value	OR (95% CI)	p-value	OR (95%CI)	p-value
Age(years)	1.112 (1.074, 1.152)	0.0001	1.113 (1.071, 1.157)	0 .0001	1.150 (1.065, 1.243)	0 .0001
Sex	1.69 (0.875, 3.263)	0.118				
ALT U/L	1.004 (0.998, 1.004)	0.156				
ASTU/L	1.012 (1.004, 1.019)	0.002	1.019 (1.004, 1.033)	0.012	0.993 (0.981, 1.006)	0.307
TB mg/dl	1.128 (0.803, 1.847)	0.354				
Hospital Stay	1.008 (1.005, 1.011)	0.0001	1.075 (1.023, 1.130)	0.004	1.084 (1.004, 1.172	0.040
LDH					2.009 (2.005, 2.014)	0.0001

Abbreviations: OR = odds ratio,
Model 1 variables entered in step 1 Age, sex, ALT, AST, TB, and hospital stay.
Model 2 Age, AST, hospital stay and LDH

Table 3. ROC curve analysis for Age, AST, and LDH.

Parameters	**AUC (95% CI)**	**Cuttoff-value**	**Sensitivity**	**Specificity**	**p-value**
LDH	0.880 (0.809-0.951)	615 IU/L	80.0%	83.0%	0.0001
Age	0.789 (0.709-0.888)	59.5 years	75.6%	71.7%	0.0001
AST	0.684 (0.579-0.789)	59 U/L	60.0%	61.3%	0.006

were raised in 58.4% and 39.0% of the subjects, respectively [12]. In another study including 1827 US patients, elevated concentrations of AST and ALT were present at admission in 66.9% and 41.6% of the patients, respectively [13]. In research conducted on deceased and recovered patients, higher concentrations of transaminases and total bilirubin were observed in nonsurvivors than in survivors [14].

The phylogenetic resemblance of SARS-CoV-2 to SAR-CoV and MERS supports the evidence of liver dysfunction in subjects with Covid-19. Previously, elevated levels of transaminases and liver impairment have been observed in critical SARS-CoV and MERS-infected patients [15-17]. The clinical data have shown that hepatic damage in most patients with Covid-19 is manifested as mild elevation (usually < 3 × ULN AST/ALT) accompanied by slightly higher bilirubin levels. Additionally, in the current study, like many others, a more frequent elevation in AST than ALT is observed [10, 12, 18]. Among the several proposed hypotheses for liver dysfunction in Covid-19, one potential mechanism is the direct cytopathic effect of the virus on biliary epithelium or hepatocytes through the upregulated expression of ACE2 receptors. Single-cell RNA-sequencing of ACE2 receptors in liver cells has shown the highest release (60%) in cholangiocytes, followed by the lowest in (3%) of ACE2 mRNA (3%) in hepatocytes and absent in other liver cell types [4, 19]. In contrast to greater ACE receptor expression in cholangiocytes, Covid-19 infection is characterized by a hepatocellular pattern of liver injury that is manifested as elevated concentrations of ALT and AST. Jaundice or some degree of cholestasis is not commonly observed even in intensive care units (ICU) patients. Additionally, viral RNA in the liver cells of dead patients was not confirmed by PCR [20]. The other possible pathophysiology implicated in hepatic injury in critically ill patients is the hyperactive immune-mediated response related to an excessive uncontrolled release of interleukins (cytokine storm), eventually culminating in multi-organ failure and acute respiratory distress syndrome.

In the current study, AST showed a significant correlation with LDH and ferritin. The upraised concentration of AST with the concomitant rise in LDH levels mirrors tissue damage linked with many disorders, including liver and lung ailments. Additionally, LDH is essentially involved in anaerobic respiration and is usually found raised under hypoxic conditions in the liver, i.e., hepatic congestion. The increased values of ferritin are indicative of the hazardous role of immune-mediated inflammation in liver injury [21]. Abnormal liver function at presentation in infection is suggestive of the fact that liver damage is not the result of medical intervention but rather a multifactorial phenomenon.

Moreover, endothelial cells of both small and large arteries have ACE expression. ACE2 (1-7) is produced by the vascular endothelium [22, 23]. In the vasculature, the ACE2/ angiotensin- (1-7)/ MAS axis has antithrombotic, antiproliferative, and vasodilatory actions. SARAS-CoV2 RNA has been found in the endothelia of many small vessels [24]. Critical Covid-19 patients had considerably higher plasma D-Dimer levels [25]. Disseminated intravascular coagulation (DIC) is a common occurrence in the early stages of the infection. Additionally, coagulation factors are produced by the liver cells, any damage to the liver will impact the coagulation process negatively.

5. CLINICAL SIGNIFICANCE

Monitoring of liver dysfunction in the early stage of Covid-19 infection could identify severe cases and contribute towards better management of the patients.

6. CONCLUSIONS

Hepatic dysfunction in Covid-19 disease at presentation is mainly manifested as mildly raised hepatic transaminases. Liver damage in subjects with Covid-19 appears to be a complex phenomenon including direct cytopathic of the virus, hyperactive immune-mediated response, and hypoxia generated by respiratory distress. Older age and raised lactate dehydrogenase at presentation can be used as predictors of severe disease in patients with Covid19.

7. ETHICAL APPROVAL STATEMENT

The Ethical Review Board, Rawalpindi Medical University, approved the project (83/IREF/ RMU/2020).

8. ACKNOWLEDGMENTS

Thanks to the entire staff of the Corona ward at Benazir Bhutto Hospital, Rawalpindi for their cooperation in the data collection for this study. No funding was received for this project.

9. AUTHOR CONTRIBUTION

All authors contributed equally.

10. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Appendix 1. Distribution of laboratory parameters at admission.

Ferritin (ng/mL)	492.88 (15-1000)
Lactate dehydrogenase	688(169-3922)
Bilirubin	
Normal	173 (94.5%)
1–2 IN	7 (3.8%)
2–3 IN	1 (0.5%)
3-5 IN	1(0.5%)
≥ 5ULN	1(0.5%)
Alanine Aminotransferase	
Normal	78 (42.6%)
1–2 IN	58 (31.7%)
2–3 IN	26 (14.2%)
3-5 IN	15 (8.2%)
≥ 5 ULN	5 (2.7%)
Aspartate Aminotransferas	
Normal	56 (30.6%)
1–2 ULN	84 (45.9%)
2–3 ULN	20 (10.9%)
3-5 ULN	15 (8.2%)
≥ 5ULN	7 (3.8%)

Appendix 2. Comparison of subjects with a normal and raised level of hepatic enzyme.

Parameters	**AST-ALT ≤ 40 (n=44)**	**AST-ALT ≥ 40 (n=138)**	**p-value**
Age(years)	52.39±16.05	57.75±15.28	0.047
Sex (male)	26 (62%)	93 (66%)	0.591
ALT U/L	21 (12-39)	57 (12-1209)	0.0001
AST U/L	30 (17-40)	61(21-630)	0.0001
TB mg /dl	0.5 (0.3-1.40)	0.6(0.3-8.0)	0.124
LDH	419 (170-936)	624 (220-3922)	0.003
Ferritin	246 (45-791)	592 (15-100)	0.001
Hospital stay	3 (1-24)	7 (1-46)	0.032
Mortality	6 (14.2%)	52 (37.14%)	0.003

Appendix 3. Comparison of variables between discharged and deceased.

Variable	**Discharged N=124 (68.1%|)**	**Deceased N= 58 (31.9%)**	**p-value**
Age	50.33 ± 14.11	67.23±11.86	0.0001
Sex	77 (62.1%)	42 (72.4%)	0.186
ALT U/L*	45.5 (12-270)	49 (17-1209)	0.116
AST U/L *	46.9 (16-317)	69.7 (21-630)	0.0001
TB mg /dl*	0.5 (0.3-8)	0.7 (0.3-2.60)	0.075
LDH *	470 (170-1622)	870 (265-3922)	0.0001
Ferritin*	357 (15-100)	671 (82-1000)	0.001
Disease severity	14/57 (24.6%)	43/57 (75.4%)	0.0001
Hospital stay*	4 (1-35)	9 (1-46)	0.001

* Mann Whitney U test

Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 61(2): 235-242 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-2)1056

Pakistan Academy of Sciences

Research Article

Exploring the Relationship between *SLC30A8* Gene Polymorphism and Type 2 Diabetes Susceptibility in District Vehari, Pakistan

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Abstract: Type 2 diabetes mellitus (T2DM) is estimated to afflict 537 million individuals globally and has reached an epidemic scale. These global estimates are to develop innovative preventive and treatment methods and to put these methods into action. To investigate if *SLC30A8* gene polymorphisms can be used to predict the onset of T2DM in residents of Punjab, Pakistan, two groups were established based on prospective follow-up of appropriate population samples. Males made up 29.6% and women 70.4% of the T2DM unit. Deoxyribonucleic acid (DNA) was separated. Polymerase chain reaction or PCR was used for genotyping, and real-time PCR was then conducted. The statistical analysis was performed utilizing the statistical package SPSS 16.0 software program. The *SLC30A8* gene genotype TT rs13266634 was linked to an increased risk of type 2 diabetes mellitus (T2DM) (relative risk — RR 1.51, 95% confidence interval — CI 1.11 – 2.05, p = 0.008). A protective benefit against T2DM was linked to the *SLC30A8* gene's CC genotype, rs13266634 (RR 0.57, 95% CI 0.35 – 0.92, p = 0.026). The T2DM group comprised 442 individuals in the District Vehari. The average age at the time of the initial screening was 56.2 ± 6.7 years. 531 individuals without diabetes were chosen to serve as controls; their average age was 56.1 ± 7.1 years. In the control group, the frequencies of single nucleotide polymorphisms (abbreviated as SNP) match the expected frequencies as per the Hardy–Weinberg equilibrium. The *SLC30A8* gene's rs13266634 polymorphism shows its correlation with the likelihood of developing T2DM and can be a potential candidate for a diabetes risk score.

Keywords: Genotype, rs13266634, *SLC30A8*, Single Nucleotide Polymorphism, Type 2 Diabetes Mellitus.

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) accounts for 537 million instances of diabetes globally. In 2021 the International Diabetes Federation (IDF) concluded 32,964,500 total cases of adults who had diabetes, indicating a 26.7% prevalence, among 123,526,400 total population of adults in Pakistan [1]. In 2022, the International Diabetes Federation appraised that 26.7% of Pakistani adults had diabetes, accounting for over 33,000,000 diabetic cases [2]. Diabetes has multiple recognized etiological causes, including genetic mutation, physiological changes, societal pressures, and unhealthy lifestyle choices. One of the most fatal diseases is diabetes, also referred to as glucose intolerance. It ranks as the fourth most deadly illness at the moment, and its prevalence is quickly increasing [3].

There are four different types: Insulin production by the pancreas is impaired in people with Type 1 Diabetes Mellitus (Insulin-dependent or IDDM), whereas, in Type 2 (Non-Insulin dependent or NIDDM), in which the body fails to react correctly to the action of produced insulin, Type 3 (gestational), and Type 4 including monogenic diabetes syndrome (Neonatal diabetes or diabetes among individuals below 25), exocrine pancreatic disease (pancreatitis, cystic fibrosis) and drug or chemically induced diabetes [4]. A chronic illness known as type 2 diabetes is brought on by improper insulin activity in the body. It is characterized by tissue resistance to insulin action, which increases levels of blood glucose. Type 2 diabetes is brought on by the pancreatic cells that produce insulin. The body receives a release of the hormone insulin from the pancreas when blood sugar (glucose) levels rise.

Received: March 2024; Revised: May 2024; Accepted: June 2024
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Approximately 90% of instances of diabetes are type 2, which is significantly more frequent than type 1. Adolescence is when it typically happens. The body generally reacts poorly to insulin, which is why the pancreas here does not produce enough of it to maintain normal blood glucose levels [5, 6]. In recent years, there has been a notable increase in the prevalence of type 2 diabetes mellitus (T2DM), a condition that has substantial socioeconomic repercussions. According to the "International Diabetes Federation", there are 537 million adult cases of diabetes worldwide among people aged 20 to 79, and by 2045, there should be 783 million cases (IDF Diabetes Atlas 10th Edition). This disorder is the cause of high mortality due to repercussions such as cerebrovascular abnormalities, amputations, cardiovascular diseases, and renal failure. The disease's complicated etiology encompasses a number of variables that affect a person's risk and prognosis, including family history, ethnicity, a poor diet, a sedentary lifestyle, obesity, and dyslipidemia [7, 8]. In terms of genetics, extensive global research has demonstrated the correlation between this illness and many genotypic variations of almost 80 potential genes. The majority of the variations found in these genes have an impact on insulin secretion, however, it's still unclear exactly how they work at the molecular level [9].

In addition to having high blood sugar, people with T2DM have the possibility of developing multiple other conditions, including heart attacks, strokes, renal failure, blindness, and amputation of lower limbs. The prevalence of diabetes is predicted to increase further, affecting about one in three people by the year 2050 [10]. One of the consistently reported risk factor SNPs to T2DM is the rs13266634 SNP found in the *SLC30A8* gene. Located on chromosome 8q24.11, the *SLC30A8* gene is made up of eight 37kb exons that encode a 369 amino acid protein, zinc transporter protein member-8 (ZnT-8) [11]. The rs13266634 is a missense SNP in the last exon of *SLC30A8* gene, where a change between the two nucleotides C/T occurs, leading to alteration between the two amino acids arginine (R) and tryptophan (W) at the position 325 (R325W). The risk C allele that encodes for arginine has shown an association with lower early insulin response to glucose and increased susceptibility to developing T2DM disease [12, 13]. While the T allele acted as a protective polymorphism against the onset of T2DM the rs13266634 C allele raised the chance of developing T2DM [14, 15]. Nonetheless, our aim in this research project is the diagnosis of T2DM patients by investigating the genotypic frequencies of rs13266634 SNP of gene *SLC30A8*, among Pakistani individuals in the District Vehari.

2. MATERIALS AND METHODS

This one-year study was conducted in 2023 among 1000 Individuals; out of these, only 442 individuals were selected for further research purposes, while the remaining 558 were excluded. Individuals were selected from DHQ Hospital of District Vehari, in the 30–79 age range, who were T2DM affected and those who were solely taking T2DM medication, and further experimental lab work was conducted in labs of the Institute of Microbiology and Molecular Genetics of Punjab University, Lahore. Patients who were smokers or consumers of nicotine, alcoholic, expected women, who were taking steroid medications, like prednisolone, and had other types of diabetes were excluded. The salting out method for the extraction was used to separate DNA from blood. The known information about the polymorphisms of the candidate genes and their correlation with T2DM guided the selection process. Additionally, potential mechanisms of their application in the pathophysiology of T2DM were considered throughout the gene selection process. The *SLC30A8* gene's rs13266634 polymorphism was detected using a polymerase chain reaction. The restriction fragment length polymorphism was then analyzed. Primer 5'-GTCAGAGCAGTCGCCCAT-3' (Forward primer, binds to the sense strand of target DNA, having 60% GC content and its melting temperature is approximately 58-60 °C) and 5'-CCTGGTCAACTGGAGATTCCA-3' (Reverse primer, binds to the antisense strand of DNA, 55% GC content and its Tm is approximately 56-58 °C) were employed to bind specifically to a target sequence with a high degree of complementarity with genotype of *SLC30A8* gene for rs13266634. 33 cycles of denaturation at 95 °C for 30 s, primer annealing at 56°C for 30 s, and elongation at 72 °C for 30 s comprised the temperature regime used for amplification. Restrictase MspI enzyme was used at 37 °C for 16 hours, which have recognition sites C↑CGG GGC↓C. Electrophoresis in 1% agarose gel was used to identify amplification and restriction products, which were then stained with ethidium

bromide. The amplification product measured 171 bp in size (Figure 1). Following restriction, products corresponding to the TT genotype, the CC genotype, and the heterozygous CT genotype were found.

2.1. Statistical analysis

The statistical software program SPSS 16.0 was used for statistical processing. The X^2 test was used to determine how well genotype frequencies matched the Hardy–Weinberg equilibrium. Using Fisher's exact two-tailed test and Pearson's Chi-Square or X^2 test, the significance of the genotype frequency differences between the T2DM group and the control group was determined. The threshold of significance was set at $p < 0.05$.

A binary logistic regression approach with a feature sequential inclusion function was utilized to create statistical risk assessment models. The genotype and allele frequencies of the two investigated polymorphisms in genes (*SLC30A8*) among the T2DM unit and the untreated or control group were analyzed. Furthermore, these frequencies were assessed independently in males and females under the age of 55 and in those over the age of 55.

3. RESULTS

With a verified diagnosis of a new case of T2DM, the T2DM group comprised 443 individuals (70.4% women and 29.6% males). The average age at the time of the initial screening was 56.2 ± 6.7 years. A total of 532 individuals (32.7% males and 67.3% females) with no history of diabetes were chosen as controls; their average age was 56.1 ± 7.1 years. The Hardy–Weinberg equilibrium predicts that the observed frequencies of SNP genotypes rs13266634 of the *SLC30A8* gene in the control group correspond to the predicted frequencies (X^2 = 0.52), see Table 1 and Figure 2.

The T2DM group's proportion of homozygotes with CC showed a substantial decrease (odds ratio (OR) 0.575; 95% confidence interval (95% CI) 0.36 – 0.93; $p < 0.026$). Therefore, it is likely that the

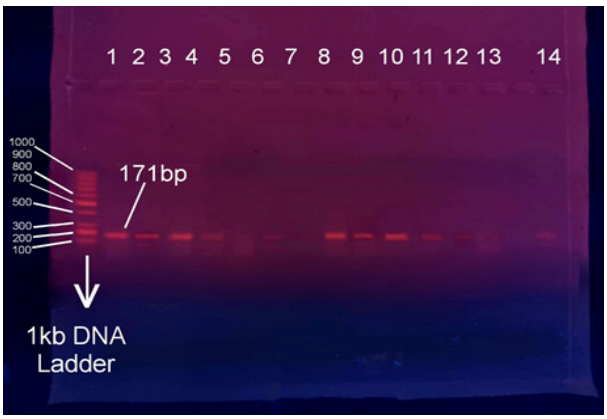

Fig. 1. Pictorial representation of gel electrophoretic results in which a 1kbp DNA Ladder was used. The product measured 171 bp in size. This image was taken under a UV spectrophotometer, making Ethidium bromide with DNA, glow and make DNA visible to the human eye.

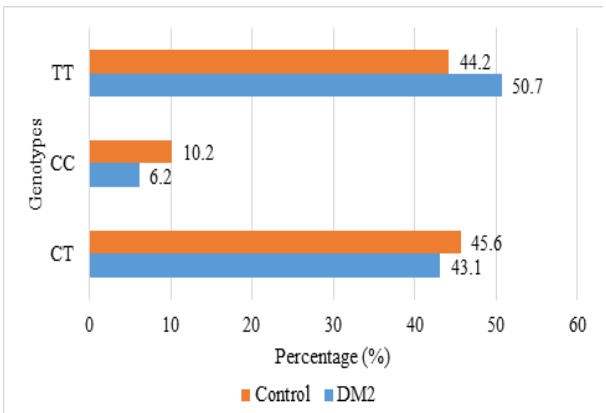

Fig. 2. Bar Chart representation of genotype frequencies of the *SLC30A8* gene's rs13266634 polymorphisms are compared between those with type 2 diabetes mellitus unit and the control group.

Table 1. Groups with and without disease of type 2 diabetes mellitus for the investigated polymorphisms' genotype frequencies.

Gene	Genotype	Group T2DM		Control group	
		n	%	n	%
SLC30A8	TT	224	50.7	235	44.2
	CC	27	6.2	54	10.2
	CT	191	43.1	242	45.6

***Note:** n is the number of individuals. Here TT genotype of the *SLC30A8* gene depicts higher frequencies in the type 2 diabetes mellitus group as compared to the genotypic frequencies of the control group for rs13266634 polymorphism. In other words, CC and CT genotypic frequencies are higher in the control group than in T2DM patients.

homozygous CC genotype of the *SLC30A8* gene's rs13266634 polymorphism confers conditional protection against T2DM. The frequency of genotypes of the *SLC30A8* gene's rs13266634 polymorphism showed statistically significant differences solely in women when groups divided by sex were compared. Compared to carriers of the other two genotypes, women carrying the TT genotype have a 1.5-fold increased risk of having T2DM (OR 1.51; 95% CI 1.11 – 2.05; p = 0.008), see Table 2 and Figure 3. There were no statistically significant differences between the T2DM and control groups when comparing the genotype frequencies of the *SLC30A8* gene's rs13266634 polymorphism, segregated by age (p > 0.05 in both groups). But when the groups were split out according to age and sex, women 55 years of age and above showed statistically significant differences (p = 0.032), as shown in Table 3 and Figure 4.

The findings of this study indicate that there were no statistically significant variations in the percentage of women aged 55 and above who were TT homozygotes or CT heterozygotes of the *SLC30A8* gene's rs13266634 polymorphism in the T2DM group as opposed to the control group (p = 0.055 and p = 0.455, respectively). In the T2DM

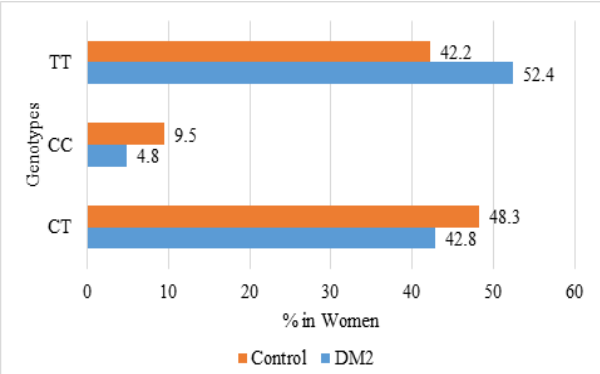

Fig. 3. This Bar chart represents a comparison of the *SLC30A8* genotype frequencies (rs13266634) in women with type 2 diabetes mellitus disease and the control group.

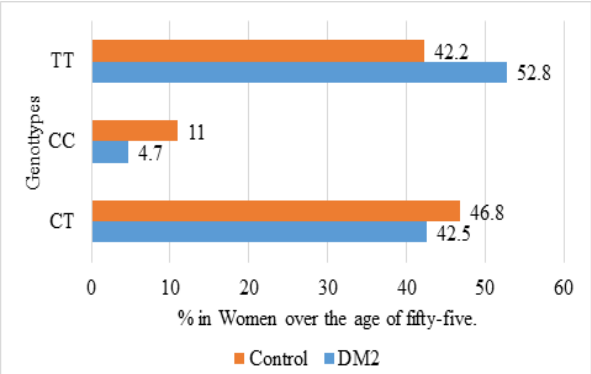

Fig. 4. This Bar chart represents a comparison of the *SLC30A8* genotype frequencies (rs13266634) in women over the age of fifty-five with type 2 diabetes mellitus disease and the control group.

Table 2. Genotype frequency of the *SLC30A8* gene's rs13266634 polymorphism, broken down by sex, in the groups with diabetes type 2 mellitus and the control group.

Genotype	**T2DM**		**Control**	
In Men				
	n	%	n	%
TT	61	46.6	84	48.5
CC	12	9.1	20	11.6
CT	58	44.3	69	39.9
Total	131	100	173	100
			p = 0.666	
In Women				
	n	%	n	%
TT	163	52.4	151	42.2
CC	15	4.8	34	9.5
CT	133	42.8	173	48.3
Total	311	100	358	100
			p = 0.008	

***Note:** Here, p denotes the significance, and n represents the number of persons. Men with type 2 diabetes mellitus disease and the control group's *SLC30A8* genotype frequencies (rs13266634) are shown in the first half of the table, which shows a significant value of p = 0.666 among men. The table's second half shows a significant value of p = 0.008 for women, allowing us to compare the genotype frequencies of *SLC30A8* (rs13266634) between women with type 2 diabetes and the women in the control group. Overall, TT genotypic frequencies are greater in both situations.

Table 3. Frequencies of genotypes and alleles of the rs13266634 polymorphism of the *SLC30A8* gene in the group of type 2 diabetes mellitus and the control group in women aged 55 years and older.

Genotype	T2DM		Control	
	n	%	n	%
TT	88	52.8	79	42.2
CC	8	4.7	21	11.0
CT	71	42.5	88	46.8
Total	167	100	188	100
			p = 0.032	

***Note:** n is the number of women over the age of fifty-five. This table depicts the *SLC30A8* genotype frequencies (rs13266634) in women over the age of fifty-five with type 2 diabetes mellitus disease and the control group.

group of women 55 years of age and above, there was a noteworthy decline in the percentage of CC homozygotes (OR 0.4; 95% CI 0.17 – 0.93; p = 0.033). Therefore, it may be concluded that the homozygous genotype CC is conditionally protective against T2DM in women 55 years of age and older and that the differences between it and the genotype at TT, while statistically insignificant, are close to threshold values.

4. DISCUSSION

To study or to overcome diabetes among the Pakistani population we explored the baseline characteristics like the genotypic frequencies of the *SLC30A8* gene's SNP rs13266634 among diabetic and non-diabetic (control) groups. Between 2021 and 2045, middle-income nations are predicted to experience the largest relative rise in the overall incidence of diabetes (21.1%), in contrast to high-income-(12.2%) and low-income (11.9%) countries. The predicted cost of diabetes-related medical expenses worldwide was 966 million dollars in 2021 and is expected to rise to 1,054 billion USD by 2045. Approximately 463 million individuals globally have diabetes, which translates to more than 10.5% of adult adults worldwide living with this illness [16]. Medical professionals also think that Pakistan's diabetes epidemic is partly caused by poor eating habits, inactivity, and an increase in obesity [17]. Over a year, the combination drug linagliptin and metformin preserved the comparable safety profile and clinically substantial improvements for glycemic control [18]. Zinc transporter ZnT-8 promotes the collection of zinc across the cytoplasm towards intracellular vesicles; zinc transporting zinc to insulin synthesis and/or storage mechanisms within insulin-secreting pancreatic β-cells may be mediated by ZnT-8. Insulin synthesis & storage depend on zinc. In terms of pathological conditions, zinc and both types of diabetes seem to have intricate relationships [19, 20]. A significant amount of illness risk can be explained by a polymorphism within the zinc transporter *SLC30A8*, which is only expressed in β-cells that produce insulin. This polymorphism also serves as proof of concept for a genome-wide strategy for the elucidation of complicated hereditary features [21]. Growing older and a longer period are linked to the prevalence of diabetes [22]. ZnT8 trafficking to the cell surface is facilitated by glucose-stimulated insulin secretion (GSIS) [23]. Zinc transporter 8 (ZnT8) serves as a prominent autoantigen, that is widely distributed on the surface of β-cells. In type 1 diabetes, this particular molecular target may protect β-cells from autoimmune assaults [24].

Since there are significant variations among human and rodent models, novel strategies include lower-frequency variants for a tool for clarifying gene functions, enabling greater comprehension of the illness and offering potential therapeutic targets. Most studies regarding the role of ZnT8 in T2DM have focused on animal models and frequent high-risk variants [25, 26]. In-depth research on the interactions between all of the underlying elements, such as gene polymorphism, is necessary to have a better knowledge of this disease [27]. The information gathered regarding the *SLC30A8* gene's rs13266634 polymorphism is in line with the findings of prior investigations. It has previously been demonstrated that variations in this gene are linked to the onset of T2DM in several populations [28 - 30]. Specifically, research conducted by Russian scientists revealed that having the T allele raises the likelihood of having T2DM (OR = 1.36), but having the C allele lowers this likelihood (OR = 0.74) [31]. There are conflicting findings on the relationship between T2DM and the *SLC30A8* gene's rs13266634 polymorphism. This could be

brought about by variations in the racial, ethnic, and regional makeup of the populations under study, as well as in the methods used for sampling and analysis. In the Chinese population, the C allele of the rs13266634 polymorphism is linked to the dysregulation of glucose and T2DM [32]. The rs13266634 polymorphism of the *SLC30A8* gene has a moderate effect on the susceptibility to type 2 diabetes, according to research on its relationship with T2DM in India [33]. rs13266634 may be a significant genetic risk factor for T2DM in Asian and European populations, but not in African people, according to many meta-analyses [34]. The protective impact of SNP rs13266634 of the *SLC30A8* genome on T2DM disease in Pakistani women 55 years of age and older is validated and supported by the results of the prospective investigation of the study [35]. Diabetes mortality can be decreased by closely monitoring blood glucose levels because the consequences of the disease are growing increasingly severe. Diabetes mellitus affects women more frequently. If we consider how long the disease has lasted, we can determine that women who have diabetes mellitus have doubled in number. Nonetheless, based on the comparison of men's and women's adherence to medication, it may be inferred that males are marginally more likely than women to take medication [36]. When it comes to following a diet, it is evident that men are more likely than women to break their diets. The incidence of diabetes is correlated with age and duration of time as aging is associated with a decline in insulin sensitivity and beta-cell function, which is crucial for regulating blood sugar levels so, that when people grow older the chances of diabetes increase. The longer the person is exposed to risk factors such as poor diet, sedentary lifestyle, obesity, and genetic predisposition, the higher the likelihood of developing diabetes as, over time, these factors can lead to insulin resistance and beta-cell dysfunction, culminating in diabetes [37]. Nonetheless, in this research project, we explored the relationship among T2DM patients and investigated the genotypic frequencies of rs13266634 SNP of gene *SLC30A8*, among Pakistani individuals.

Clinical significance of the results The SNPs that were shown to be likely T2DM markers had results of linkage with T2DM, were distinct from those of the Pakistani population, and were also examined for the inaugural time. It is thought to be possible to include the examined gene variations in the disease's risk scale model. Thus, the *SLC30A8* gene's rs13266634 polymorphism, which has been linked to the occurrence of T2DM, can now be included in the T2DM risk meter.

5. CONCLUSIONS

The *SLC30A8* gene's rs13266634 polymorphism has demonstrated its correlation with the likelihood of developing T2DM and thus is a viable option for inclusion into the T2DM genetic risk meter. The *SLC30A8* gene's genotype TT of rs13266634 was linked to a 1.5-fold increased risk of T2DM (RR 1.51, 95% CI 1.11 – 2.05, $p = 0.008$) in Men, Women, and even in specific groups of women over the age of fifty-five. The T2DM group's proportion of homozygotes with CC showed a substantial decrease (odds ratio (OR) 0.575; 95% confidence interval 0.36 – 0.93; $p < 0.026$). Therefore, it is likely that the homozygous CC genotype of the *SLC30A8* gene's rs13266634 polymorphism confers conditional protection against T2DM. To completely describe the underlying, causative variant, and frequently the causal gene itself, a lot of obstacles still need to be overcome. The future appears promising for the creation of innovative treatments and diagnostics for diabetes of the type 2 variety and its associated characteristics, nevertheless, if progress is made on these fronts.

6. ETHICAL APPROVAL

Ethical approval was obtained from the Microbiology and Molecular Genetics, Institutional Research Ethics and Biosafety Committee under reference number D/227/MMG.

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

8. ACKNOWLEDGMENT

We thank the Institute of Microbiology and Molecular Genetics for carrying out this investigation.

9. AUTHOR'S CONTRIBUTION

FG performed research work and did data analysis with the writeup, SR did proofread and helped in data analysis.

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