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Review Article

Potential Role of Extracellular Matrix and its Components in Cancer Development and Progression

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Abstract: Cancer occurs due to unregulated multiplication of cells. Extracellular matrix (ECM) proteins come in a huge variety, and each one has unique biochemical and biophysical characteristics that affect the phenotype of cells. To ensure tissue homeostasis, the ECM undergoes continuous deposition, remodeling, and degradation from early development until maturity. In order to govern cell behavior and differentiation, the ECM's composition and structure are spatiotemporally controlled. However, when ECM dynamics are dysregulated in any way, illnesses like cancer can arise. Collagen is a major component involved in ECM regulation but after cross linking with each other, it initiates ECM stiffness, loss of cell contacts and cell geometry. Due to which most of the regulators including the Transcriptional coactivator with PDZ-binding motif (TAZ) and Yes-associated protein (YAP) are inhibited and cause extensive cell proliferation and tumor metastasis. Proteases like Metalloproteinases degrades collagen and other proteins that leads to ECM break down and cancer progression. As cancer spreads, the stress and pressure on cells increases which damage arteries and capillaries causing hypoxia. Hypoxia inducible factors take advantage of the situation and enhance invasiveness of cancer cells. This stress generated by tumor cells in their surrounding causes dysregulation of ECM matrix. Finding strategies to study the relationship between mechanical stress in tumors and their destructive behavior is vital for cancer research.

Keywords: Cancer Development and Progression, Tumor Microenvironment, Extracellular Matrix.

1. INTRODUCTION

Cancer refers to the condition in which cells of the specific part of the body proliferate uncontrollably, invade in neighboring cells and organs, and destroy the healthy cells. Sometimes those cells localized in a specific part called benign tumor but sometimes it spread. This spreading of tumor cells from one part to other areas of whole body is known as metastasis or tumor progression [1]. Multiple symptoms indicate tumor progression including lump formation, unexplained bleeding etc. [2]. Cancer became the leading cause of death and almost 90% deaths are due to cancer progression worldwide. This death rate is increasing annually though there are great advances in the treatment of cancer from targeted antibiotic therapy to chemotherapy [3]. Numerous researches are ongoing to explore novel therapeutic components to target cancerous cells either by ceasing their proliferation or by eradicating them [4]. Basically, Tumor microenvironment (TME) is involved in affecting cancer progression that not only consist of tumor but also the non-cancerous cells that include immune cells, endothelial cells, adipocytes, interstitial cell, extracellular matrix etc. TME and especially ECM has proved to be the most advantageous niche for cancer cells enrichment [5]. Extracellular matrix (ECM) is the non-cellular component of tissue and secreted by the cells for the sake of biochemical and structural support. ECM comprises of polysaccharides, proteoglycans, proteins, water and all of these components help in survival, differentiation and functioning of that particular tissue [6]. There are several proteins involved in the formulation of extracellular matrix that are classified into two

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major categories, glycosaminoglycan and fibrous proteins. Collagen, elastin, fibronectin and laminin are included in fibrous proteins while hyaluronic acid, heparin sulfate and chondroitin sulfate are example of glycosaminoglycan. These proteins are evenly distributed in extracellular matrix in the form of crosslinked meshwork [7]. There is a strong relationship between ECM and cancer progression. Cancer cells are responsible for the rigidity of extracellular matrix and in repay the rigid ECM changes the structural characteristic of cancer cells. The connection between these two activates multiple signaling and regulatory pathways. So, it's very important to understand the basic phenomenon that would assist to discover more therapeutic targets for treatment of cancer [8].

1.1. Functioning Mechanism of ECM

All the components of ECM functions in an orderly manner to maintain the physical, structural and biochemical properties of cells and tissues that are crucial to regulate cell behavior. The physical function of ECM is to retain the porosity, rigidity, insolubility of membrane and integrity of tissues [9]. As shown in the Figure 1, ECM provides anchorage site to tissues that helps them in migration from one compartment to other. It also implicates biochemical properties by acting as signal reservoir and initiate signal transduction pathways by interacting the cells with their microenvironment. It can particularly bind to different growth factors and serve as signal co-receptor. It also helps the cell to present signal receptor on their surface and promote cell adhesion. ECM also initiate cell signaling pathways by operating as precursor of signaling fragments. Cells

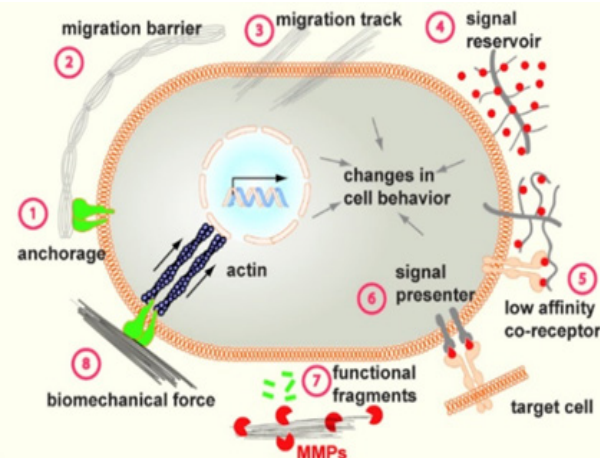

Fig. 1. Functioning mechanism of extracellular matrix.

recognize the biomechanical ability of ECM that is involved in maintaining stiffness of tissues [10].

One of the most interesting features of cell and extracellular matrix is that their relationship is reciprocal. Cells are rearranging various components of ECM to carry out signaling and biosynthetic pathways while ECM regulates and maintain cell's normal behavior and changes in any component of ECM leads to change behavior of other cells which causes different abnormalities or disease like cancer [11].

1.2. Components of ECM

The ECM is made up of a variety of proteins, which result in the varied structures and characteristics that it possesses. Laminin, fibronectin, proteoglycans, and collagen are the ECM's primary building blocks. There are multiple subtypes of these blocks that can further explain their role in the general structure and characteristics of ECM, even among these ECM components (Figure 2). Diverse subtypes of ECM molecules bestow various functions that proved to be crucial for proper operation of entire body since structure determines function**.** Table 1 shows the general components of extracellular matrix and few of those components are comprehensively discussed below:

1.2.1. Collagen

With 28 distinct subtypes identified, collagen is the most important element of the ECM and the most prevalent protein in human tissue. Each kind is made up of right-handed triple helices that are twisted into left-handed helical chains either in form of heterotrimers or homotrimers. The Gly-X-Y motif is present in a wide collection of proteins known as the collagen superfamily, where proline or hydroxyproline are often used for X and Y [13]. The tiny glycine and interchained hydrogen help to stabilise the large proline and right-handed helical shape. Depending on where it is located in the tissue, each collagen fibre is composed of different subtypes of collagen. Kind I collagen, the most prevalent type of fibrillar collagen, is present in connective tissues including skin, bone, tendon, and cornea. Organ development and wound healing are two processes in which collagen I plays a significant role [14].

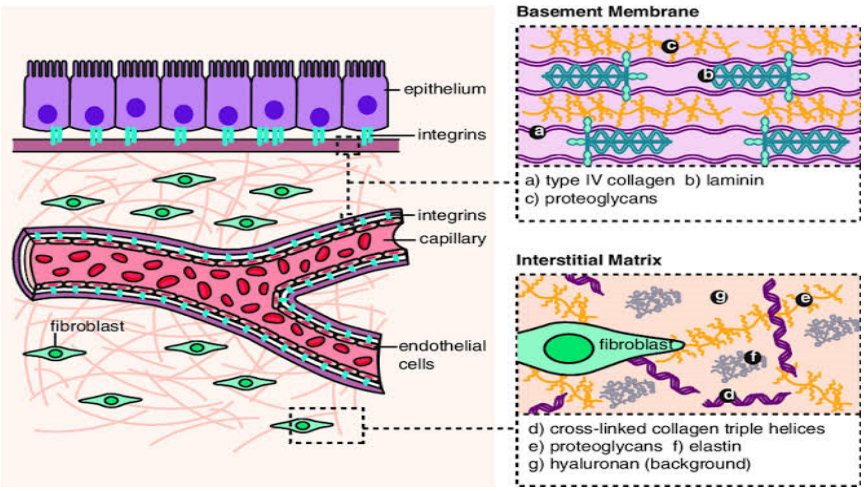

Fig. 2. Arrangement of certain ECM molecules in the basement membrane and interstitial stroma. The distinctive elements of the extracellular matrix are displayed in Panel A (top). The organisation of various proteoglycans, collagens, fibrone [46].

Precursors are created before any fibrillar collagen is generated. The triple helical helix is put together by the chains in (RER)rough endoplasmic reticulum. For initiation of the triple helical helix, lysine and proline are hydroxylated followed by the glycosylation of the molecule. Golgi apparatus is where the procollagen is subsequently processed for cellular export [15]. In the ECM, procollagen is either processed before or after it is secreted.

Specific matrix metalloproteinases (MMPs) break the C terminal propeptide off, and if it is not removed, collagen becomes very soluble and is unable to form fibrils [16, 17]. The N-propeptide in first three collagen types are cut off, but they are left in type V, type XI, and other fibrillar collagens. This doesn't influence fibril production but changes the fibril's shape and diameter. Through steric hindrance and charge interactions, the N-propeptide of type XI and V collagens stick out from the spaces among the collagen molecules to inhibit lateral growth [18]. Currently, it is thought that type XI and type V collagens nucleate and regulate synthesis of collagen fibrils. Despite relatively modest levels in the overall collagen composition of most tissues, it was demonstrated that in case of mice, collagen V deletion results in fibril disassembly. Once produced, the microfibrils may join forces with other microfibrils to expand into bigger fibres. Other ECM proteins mediate this process.

Decorin and biglycan are examples of small leucine rich proteoglycans (SLRPs) that include motifs (collagen binding) that allow to control fibre development, content, shape, and size [20]. Fibril-associated collagens with interrupted helices

Table 1. General components of ECM and their functions [12].

Components of ECM	Functions
Proteoglycans: (Aggrecan. Betaglycan, Decorin, Perlecan Syndecan)	• Facilitate cell movement during tissue morphogenesis and repair; Control the actions of released proteins and play a significant part in cellular chemical communication.
Glycosaminoglycans: (Hyaluronan Chondroitin Sulfate, Dermatan Sulfate, Heparan Sulfate, Keratan Sulfate)	• Co-receptors that work in tandem with traditional cell-surface receptor proteins to bind cells to the ECM and to start their reaction to some extracellular signal proteins; Enables the blood and tissue cells to rapidly diffuse nutrients, metabolites, and hormones; Helps the matrix endure compressive stresses by forming hydrated gels.
Fibrous proteins: (Collagens, Elastin, Fibronectin, Laminin)	• Give the matrix more resilience, and strength; Activate intracellular signalling pathways through influencing cell behaviour and survival, development, shape, and polarity of cells.

(FACIT) don't result in the formation of fibrils and stay connected to collagen microfibrils surface, are another subfamily of collagen. Their main job is to attach to other ECM proteins including proteoglycans and SLRPs and facilitate the creation of higher-order structures. Lysyl oxidase (LOX) further stabilises the supramolecular collagen assembly, which results in improved mechanical qualities all around. Collagen's high tensile strength is a result of the covalent cross-linking of its N and C terminal ends by the enzyme LOX both inside and between microfibres [21].

There are network-forming collagens such type IV, type VIII, and type X besides fibrillar and FACIT collagens. By the help of 7S N-terminal domain, the type Collagen IV assembles into a tetramer. A hexamer is formed by interaction between C-terminal domain of two Collage IV molecules, NC1. The basal lamina and the interstitial stroma are divided by a stable collagen network that is made possible by collagen IV's two domains [22]. The basal lamina contains additional ECM proteins such laminin, nidogen, and perlecan that fortify this barrier and help to preserve the body's cellular order. The role of other ECM proteins including proteoglycans, laminins, and fibronectin cannot be overlooked, even though different forms of collagen are capable of constructing distinct kinds of structures that serve as foundation of ECM construction. They have a significant impact on the extracellular matrix's chemical and physical characteristics through, for example, their chemical properties and binding motifs that bind to growth factor. Moreover, they act as a connection between the cells and the ECM [23].

1.2.2. Proteoglycans

Some proteins that form covalent bonds to glycosaminoglycans (GAGs) are known as proteoglycans. Long chains of repetitions of negatively charged disaccharides, known as GAGs, can be made of chondroitin/dermatan sulphate, heparin sulphate, keratin sulphate or hyaluronan [24]. Proteoglycans are capable of sequestering cations and water by the negative charge of these GAGs, which provides them their space-filling and lubricating properties. Only those proteoglycans that are located in transmembrane, in extracellular and pericellular area will be enclosed in this review [25]. Syndecans makeup four of the thirteen transmembrane proteoglycans. These are proteins that considered to function by way of co-receptors. 3 domains are present in syndecans i.e. ectodomain, transmembrane domain, and an intracellular domain. The ectodomain is found to be linked to the GAGs, which are generally heparan sulphates and can be easily shed by the action of MMPs, [26]. Because syndecans' ectodomains are inherently disordered, there may be an interaction with a wide range of other molecules to execute a variety of natural tasks. Its actions include binding to morphogens and growth factors, assisting exosome absorption, and performing as co-receptor for tyrosine kinases receptor [27].

Perlecan is the most important proteoglycan located in pericellular region of basement membrane. It is basically a heparan sulphate proteoglycan-(HSPG) that consists of a number of domains, each with a unique set of activities and binding sites. These sulphates can attach to a wide range of substances, including collagen, growth factors, and growth factor receptors. The binding of Perlecan connects nidogen, collagen IV and laminin in basement membrane for strengthening of the basement lamina [28, 29].

Hyalectans and SLRPs are two different types of proteoglycans that are present in the extracellular area. Hyalectans have the same structure as lectins, with GAGs linked between the N and C terminal ends of the N terminal, which binds hyaluronic acid, and the C terminal, which binds lectin. Aggrecan, Versican, Neurocan, and Brevican are four different genes that encode hyalectans. While brevican and neurocan are present in CNS, aggrecan can be mostly found in bone cartilage and brain [30].

Versican, on the other hand, is present in practically all organs and tissues' ECM. It might behave as molecular link concerning the extracellular matrix and cell surface. It has been demonstrated that Versican binds to fibronectin and type I collagen, both of which are integrin substrates. Versican sequesters fibronectin from the cells integrins after binding to the RGD motif of fibronectin, which results in a lack of cell adhesion [31]. The 18 different gene products that make up the SLRP family, each of which has several splice variants and processed forms, make up the biggest family of proteoglycans. These proteins feature a leucine-rich repeat-dominated central region and

a relatively small protein core (LRRs). They are expressed in the ECM as different tissue types grow, which suggests that they play a crucial role in controlling organ shape and size throughout homeostasis and embryonic development. Along with other proteoglycans, the SLRPs biglycan and decorin, which include motifs (collagen-binding), control the assembly of collagen fibres [31].

1.2.3. Laminin

The basal lamina or certain mesenchymal compartments frequently contain laminins, which are trimeric glycoproteins made up of γ chains. Although potentially 60 different laminins might be produced by combining the 12 mammalian α, β, and γ chains, thus yet only 16 combinations have been found [12]. The size of the α chains is between 200 and 400 kDa, whereas the size of the β and γ chains is between 120 and 200 kDa. The size of a trimmer can then range from 400 to 800 kDa. Laminins emerge as molecules in cross shape under rotating shadowing-electron microscopy. Its long arm is made up of three chains, which create a -helical coiled coil structure, while each of the three short arms consists of a single chain [32].

Phenotypic maintenance, migration, adhesion, differentiation, and apoptosis resistance of laminins are all cell type-specific. Laminins can establish an active association among the cell and ECM by binding to integrins. To enable the activation of different signalling cascades and intracellular regulation of cytoskeleton, distinct heterotrimeric-laminins will have unique integrin heterodimers-binding companions [33]. Basement membrane is thought to mature when collagen IV is deposited there, which is important for structure stability in development. Laminins bind to collagen IV, although the precise process by which they do so is yet unknown. Initial research suggested that nidogen acts as a bridge between two networks existing in basement membrane by binding to laminin via collagen IV and the LE domains of the γ chain [34, 35]. Nidogen might not be the main link linking laminins and collagen IV, according to recent studies. Heparan sulphates have been found to directly mediate the interaction between laminins and collagen IV. Thought to mediate this function, perlecan was genetically deleted in mice, but this did not cause collagen IV depletion. Agrin, another pericellular HSPG, is now thought to act as a compensatory candidate. According to this concept, the laminin network that contains nidogen as well as the 7S and NC1 domains of collagen IV would all bind to perlecan and agrin [36].

Laminins are essential for assembling the basement membrane and interacting with cells of the ECM. Laminin polymerization appears to be the earliest step in the formation of the basement membrane, according to recent research. Indeed, the failure of basement membrane construction caused by genetic ablation of either γ1 chain or β1 chain evidenced to be lethal. While proteoglycans, collagen and hyaluronic acid make up the majority of ECM's structural elements, laminins are chemicals that allow cells and ECM to interact [37, 38].

1.2.4. Fibronectin

A multi-domain protein called fibronectin connects the cell to the ECM by interacting with the numerous previously mentioned ECM elements. It contains a single gene that encodes it, but due to alternative mRNA splicing in humans, it has 20 isoforms. In the ECM, fibronectin creates a fibrillar network similar to collagen [12]. The two cysteine disulfide linkages that fibronectin normally produce as dimer outside the cell, are essential for it to accumulate in a fibrillar manner. The binding to α5β1 integrins via an RGD binding motif and a synergy site on the fibronectin molecule mediates fibronectin matrix building [39, 40].

The unfolding of soluble secreted fibronectin is done by these integrins, exposing hidden sites for more binding of fibronectin molecules to create the fibrillar network. It has been demonstrated that fibronectin fibril production is inhibited by anti-fibronectin and anti-integrin antibodies. Fibronectin are present at cell surface in high concentrations as a result of integrin clustering that is brought on by fibronectin binding. Through each molecule's N terminal assembly domains, this process increases the contacts between fibronectin and fibronectin [41].

The actin-cytoskeleton causes fibronectin molecules to alter their shape once fibronectin is anchored to surface of cell by help of integrins. As a result, obscure binding sites for, heparan sulphates, fibronectin, collagen, heparin and

supplementary proteins of matrix will become visible in C terminal regions of fibronectin [42]. The network of fibronectin develops and becomes insoluble by robust non-covalent protein-protein connections, however supplementary ECM proteins may promote mature lateral contacts between the fibrils. The comparatively flimsy binding sites are stabilised at certain places by these interactions. The turnover of fibronectin matrix, however, has mostly gone unstudied [43].

Fibronectin has been linked to a number of roles, such as a part in the assembly of collagen type I, because of the numerous binding sites, it possesses for other ECM proteins. It has been demonstrated that collagen fibrils do not accumulate in the absence of fibronectin, pointing to a function for fibronectin in collagen synthesis. However, given that recent research has also suggested that collagen has a function in promoting fibronectin assembly, this interaction may turn out to be reciprocal [44, 45].

2. ECM AND CANCER

2.1. ECM Molecule Dysregulation in the Advancement of Cancer

The significance of ECM in controlling cell proliferation, cell migration and apoptosis has changed how cancer is traditionally viewed. A tissue-specific microenvironment that is crucial to the development of tumor is created by the precise orientation and arrangement of ECM elements microscopically [47]. It is now known that in addition to ongoing active remodelling, the ECM also triggers pharmacological and biophysical signals that affect cell adhesion and migration. Small changes to the homeostasis of the microenvironment can significantly influence the proliferation rate of cancer cells. Collagen, the principal component of the extracellular matrix influences the basic functionality of the matrix. The loss of ECM homeostasis can, in fact, be caused by alterations in the deposition or degradation of collagen [48, 49].

In a dynamic interaction between the microenvironment and resident cells, the surrounding ECM experiences major architectural changes as the tumor cells proliferate. The alterations, including the elevated production of fibronectin and collagens I, III, and IV, indicate that the extracellular matrix and tumor cells must continually engage for the tumor progression. Figure 3 shows the normal regulation of tissue homeostasis which gets disturbed when tumor cells exert pressure on the normal cells, and is more facilitated by the collagen deposition. They form cluster like structure nearby tumor cells and help tumor metastasis [1, 50, 51]. By interrupting with cell polarity and cell-cell adhesion, increased matrix protein deposition accelerates the evolution of tumor by boosting growth factor signalling. The precise part that collagen deposition plays in the development of tumor is complex, though. Recent research has demonstrated that increased collagen cross-linking and deposition promotes the growth of tumor through increasing integrin signalling. However, it is intriguing to note that fibrillar collagens I and III reductions to encourage harmful performance, demonstrating that the biomechanical stresses created by accumulation of collagen is equally advantageous and detrimental consequences on tumor formation [52, 53].

The process of collagen crosslinking is possible by both enzymatic and non-enzymatic ways. Amine oxidase enzymes, LOX family in

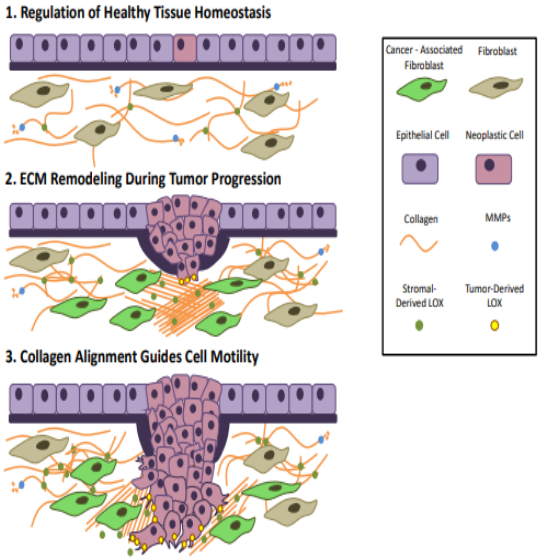

Fig. 3. How the ECM changes when cancer develops and spreads. (1) Rapid proliferation of epithelial neoplastic cells stresses the basement membrane. (2) Because of mechanical stress, the basement membrane swells. Collagen is being deposited more heavily to nearby cancer-related fibroblasts. Collagen is aligned by lysyl oxidase (LOX), which is produced from stromal cells. (3) Neoplastic cells pierce the membrane and move along collagen-aligned fibres [1].

particular, modulates collagen cross-linking in a regulated manner [54]. The production of LOX by the primary tumor cells induces the cross linking of the collagen and elastin, hence enhancing the stiffness of the matrix and the total volume of the adjacent ECM.

Increased ECM stiffness promotes the formation of focal adhesions and cell motility, by stimulating integrins and intensifying cytoskeletal stress induced by Rho. Clinical studies have linked fibrosis, elevated collagen cross-linking and an enhanced probability of cancer metastasis to increased LOX activity. Additionally, increased LOX activity had been observed to promote cell contractility, actin polymerization and migration on the invasive borders of tumors, creating a passageway to follow for succeeding tumor cells [55, 56].

During tumor metastasis, visualisation of the surrounding epithelial tissue has shown localised matrix structure and alignment along the leading edge of invasive tumors. Indeed, it has been shown that local cell invasion of these tumors is directed along collagen fibres that are aligned, which suggests that collagen fibre linearization promotes tumor invasion [57]. It can be said that these closely spaced fibres serve as conduits for spreading cancer cells to leave the tumor. Breast cancer serves as a notable example of collagen alignment during tumor propagation. Despite the fact that epithelial tissues' collagen is often knotted and chaotic frequently, the tissue that surrounds breast tumors becomes thicker, stiffer, and perpendicular to the tumor's border [58]. A recent study indicates that the architecture of the matrix Fibers reduces the protrusions along the collagen fibre, which in turn lessens the distance covered by the migrating cell and boosts the efficiency of tumor migration [59].

In ECM, increased levels of the hyaluronic acid glycosaminoglycan are correlated with greater risk of malignancy and poor prognosis, just as collagen and LOX that are essential in determining the compressive properties of many biological tissues [60, 61]. The ideal biophysical properties for tissue homeostasis result from the interplay between the tensile strength provided by collagen and the compressive compliance conferred by hyaluronic acid. Hyaluronic acid has been identified as both an induction signal and migratory substrate for mesenchymal transition [62]. Hyaluronic acid is frequently utilized as a biomarker for prostate and breast cancer. Increased LOX and collagen levels directly improve stiffness of ECM and physically drive cell proliferation and motility; however, it is yet unknown how hyaluronic acid contributes to cancer spread. However, its dysregulation can act as a vital indicator for cancer invasion and metastasis [63].

2.2. Mechanotransduction is Mediated by Protein Unfolding

ECM signalling is a critical biological mechanism which promotes cell division, cell proliferation, and prevention of apoptosis. In essence, a cell cannot survive if it cannot perceive its mechanical surroundings. Numerous investigations have revealed that via chemical signalling molecules, such as metabolic precursors and growth factors, cells are able to sense their surroundings [64]. It is thought that cells use lamellipodia to physically probe their surroundings and that integrin-based focal adhesions, which feel the mechanical feedback and resistance of their environment and set off an intracellular signalling cascade, sense ECM stiffness. The actin cytoskeleton is thought to be responsible for cells' capacity to explore their surroundings because it prevents polymerization of F-actin from inhibiting cells' ability to generate force, which has biological impact comparable to cells plating on a soft substrate [65]. In particular, contractile actin bundles and their upstream regulators, such Rho-associated protein kinase (ROCK), that are required for cells to involuntarily perceive their surroundings, are what give cells the ability to generate internal forces. While it is evident that mechanical stiffness significantly influences cellular behaviour, the mechanism by which mechanical stress is transduced into gene transcription remains poorly understood [66].

Recent studies have shown how crucial protein unfolding is for transmitting the mechanical force that the ECM exerts. In fact, it has been demonstrated that during force transmission, a major molecule in focal adhesion complexes, talin that connects focal-adhesions to actin cytoskeleton, mechanically unfolds [67]. When condensed to focal-adhesion complexes bound to talin, deletion in liver cancer 1 (DLC1), a negative regulator of cell contractility and RhoA, impacts cell's behaviour. When the talin's R8 domain was mechanically clamped, molecule's mechanical unfolding was

blocked, disrupting DLC1's downstream signalling and, as a result, disrupting cell behaviour [68].

Additionally, every rod subdomain of talin is capable to get unfold over a physiologically significant range of forces (10-40 pN), according to single molecule force microscopy. The mechanical stability of the talin rod bundles might potentially be impacted by a small number of single point mutations, given that the stability range of talin subdomain within the focal adhesion complex is dependent on minute structural changes [69]. These mutations may cause cellular responses to be misinterpreted in response to ECM signals. The performance of cancer cells in tumor microenvironment may be affected by incorrect interpretations of the ECM, which may result in deactivation of DLC1, enhanced cell migration and cell contractility [70].

2.3. TAZ and YAP Mechanotransduction in the Development of Cancer

YAP (Yes-associated protein) and TAZ (Transcriptional coactivator with PDZ-binding motif) are powerful regulators of cell survival and proliferation, and they are essential for controlling differentiation of cells, self-renewal of progenitor cells and development of organs. The YAP/TAZ proteins actively move back and forth between the cytoplasm and nucleus throughout these pathways. YAP/TAZ proteins regulate certain signaling cascades in the cytoplasm, such as the Wnt signaling pathway, in a relatively inactive manner [71]. While, they easily interact with DNA-binding transcription factors in the nucleus, notably those belonging to the TEA domain (TEAD) family, to control the gene expression linked to cell proliferation, a crucial cancer-related characteristic. The accumulation of YAP/TAZ in the cytoplasm following pharmacological inhibition suggests that the major role of YAP/TAZ is the control of gene transcription [72]. Notably, YAP/TAZ activity is suppressed when a cell separates from a substrate, indicating that the F-actin cytoskeleton and mechanical-force may be able to control how quickly YAP/TAZ travels to the nucleus. Furthermore, researchers have observed that the YAP/TAZ nuclear transport and the associated physiological processes are strongly regulated by cell-spreading geometry and matrix elasticity in mammalian systems. All of these findings point to a direct chemical pathway connecting mechanical force with malignant cellular behavior, cell signaling (cytoskeleton mediated) and coupling focal adhesion of mechanical stiffness to the YAP/TAZ pathway to cause tumor invasion and metastasis as described in Figure 4 [73].

There are several putative proteins and routes that might mediate nuclear translocation of YAP/TAZ proteins, even if cytoskeletal stress is sufficient for this to happen. The heparan sulphate proteoglycan agrin, for instance, is well renowned for its specific functioning in the development of neuromuscular junctions during process of embryogenesis [74]. Recent studies have raised the possibility that agrin may potentially behave as ECM sensor, stabilizing focal adhesions and facilitating nuclear translocation of YAP/TAZ protein via the muscle-specific kinase (MuSK) and lipoprotein-related receptor-4 (Lrp4) pathway [75]. The Hippo tumor suppressor pathway is inhibited by the activation of MuSK and Lrp4 by agrin, which eventually results in an increased YAP/TAZ nuclear translocation. It has been demonstrated that agrin depletion stimulates YAP's inhibitory phosphorylation, which forces nuclear YAP to stay in the cytosol. On the other hand, YAP activation was only required for the further delivery of agrin into cells cultivated on flexible matrices. Together with modifications in actomyosin contractility, a number of junctional proteins comprising members of the Angiomotin (AMOT) family of proteins, control protein YAP/TAZ. It has been demonstrated that AMOT proteins directly attach

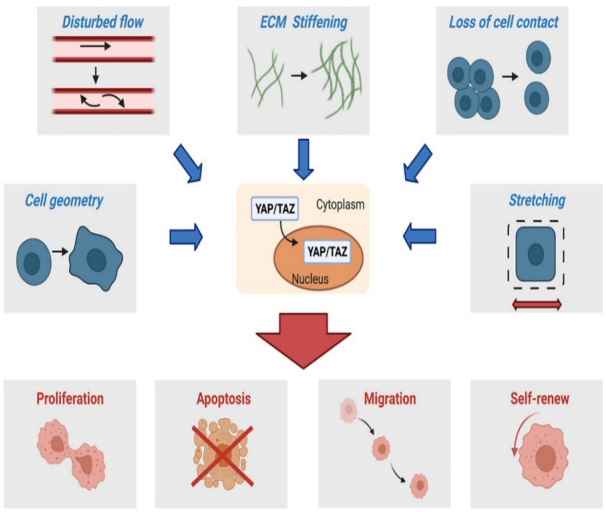

Fig. 4. TAZ and YAP Mechanotransduction in the development of cancer [76].

to YAP, preventing it from functioning. In order to break apart YAP: AMOT complexes and allow YAP to exit its inhibitory state and go into the nucleus, F-actin competes with AMOT for binding [77]. It's interesting to note that depletion of agrin increased YAP: AMOT binding, that eventually caused YAP activity to decline. Recent research further shows that Ras-related GTPase, Rap2 is a crucial intracellular-mediator that affects YAP/TAZ nuclear translocation via transducing ECM rigidity signals [78].

At low stiffness of ECM, Rap2 has been shown to attach and trigger MAP4K7, MAP4K6, MAP4K4 and ARHGAP29, that stimulates LATS1a and LATS2 though blocking nuclear translocation of YAP and TAZ. These results showed that YAP/TAZ activity modulation and ECM sensing are important functions of proteins that are superficially unrelated, such as Rap2 and agrin [79, 80].

2.4. Tumor Initiation and Migration Mediated by the ECM

Their capacity to travel across surrounding tissues and organs, penetrate the neighboring basement membrane is a critical characteristic of carcinoma and other cancer cells. This dense, cross-linked extracellular matrix serves as an anchor for epithelial cells to the surrounding connective tissues and significantly hinders their movement [81]. Nevertheless, as cells must move across the body during the homeostasis of healthy tissues, cancer cells have devised many techniques to circumvent the collagenous barrier [82]. The use of mechanical force is one such approach. The breaking of the basement membrane has increasingly been attributed to mechanical force as a compelling cause. The surrounding basement membrane limits the spread of epithelial cancer cells in terms of space. The proliferation of cancer cells significantly elevates the mechanical stress along the membrane resulting in rupture and permitting cells to escape their environment [83, 84].

Using protruding, F-actin-rich subcellular structures called as invadopodia, anchor cells invade membranes as a different type of membrane navigation. Indeed, leading invasive cells prolong a solitary protrusive arm into basement-membrane, as seen by electron micrographs of invasive tumors. The membrane fissure spreads after the invadopodia's first breach, enabling succeeding cells to cross the collagen border [85, 86]. Elevated quantities of collagen type IV degradation products were also discovered around these breaching locations, suggesting a potential third component of cancer cells migration. The widely held belief that proteases were solely responsible for the breakdown of the basement membrane has given way to an increased concentration of MMPs along the basement membrane [87]. Staining the membrane during invasion demonstrates that laminin and collagen IV are really only partially destroyed by the invadopodia. These findings suggest that rather than facilitating direct invasion, MMPs may contribute to the matrix's softening or to the first rupturing of the basement membrane [84].

2.5. Metalloproteinases (MMPs) in the Development of Tumors

MMPs have a multifaceted function in cancer cells invasion; they not only facilitate the degradation of ECM barriers in the surrounding region but also release active growth factors and promote tumor angiogenesis (Figure 5). The cell surface receptors of integrin family are recognized to be the primary mechanism by which the ECM stimulates cell proliferation [88]. However, it has been shown

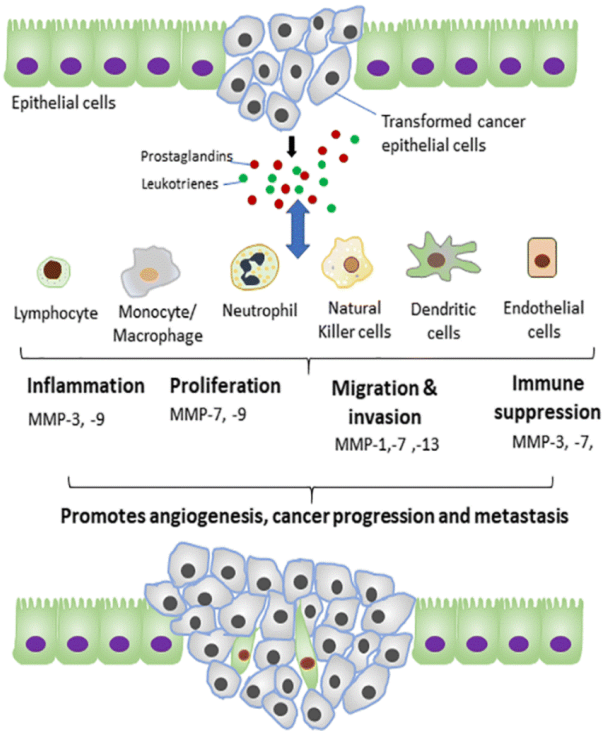

Fig. 5. Various types of Metalloproteinases involved in development of cancer [89].

that some binding sites of ECM obligatory for cell survival and proliferation are "cryptic" or partially concealed within ECM. By destroying and relaxing surrounding collagen, MMPs just reveal these covert binding sites, that enable integrins to communicate with matrix on the cell membrane directly.

MMP-mediated collagen degradation reveals signaling constituents buried within ECM in count to reduce physical barriers and displaying cryptic binding-sites. Different growth factors are inactive when they are embedded in collagen, but they become active when the ECM breaks down, allowing them to interact with their target receptor [88]. For instance, it is known that transforming growth factor β (TGF-β) active form is released during MMP-2 induced ECM breakdown. TGF- β is capable to control cell proliferation, immunological response, and invasion when it is released. In practice, MMPs not just amend the surrounding ECM to promote cell migration physically, but also release growth factors and expose cryptic binding sites, which promote the emergence of a favorable milieu for tumor formation [90].

Despite MMP-induced angiogenesis, the tumor's vascular networks are typically disordered, with inter-capillary distances commonly surpassing the oxygen diffusion limit. As a result, hypoxia (the condition oxygen level in cells decreases) is a characteristic of cancer. Research on the partial pressure of oxygen in tumors indicates a distinct association between weakly oxygenated tumors and increased malignancy [91]. Cancer cells are capable of enduring oxygen-derived environments by changing the transcription of several genes linked to angiogenesis [92]. It is well recognized that hypoxia-inducible factors (HIF) are an important role in controlling this intracellular cancer cell response to hypoxia. HIF-1α, a transcription factor belonging to the HIF family, has been linked to higher MMP and collagen formation, according to recent research [93]. It is significant to note that HIF-1α has the ability to promote LOX accumulation, which eventually stiffens the adjoining matrix. Last but not least, it has been demonstrated that HIF-1 activates transcription factors linked to the epithelial-mesenchymal transition (EMT), a phenomenon involved in decreasing the polarity and adherence of cells to one another, enhancing the invasive behavior of cancer cells [94, 95].

Unfortunately, throughout clinical studies, the majority of treatments that explicitly target MMP activity had subpar results. There are a few plausible causes for the subpar clinical results. First of all, late-stage cancer patients were those chosen to get MMP-inhibiting medicines. MMPs are recognized to contribute to the beginning and development of tumors, as was previously mentioned. MMP inhibitors could work better in people who are at an earlier stage of the disease [96].

2.6. Mechanical Stress's Effects on Tumor Growth and Treatment

As cancer spreads, structural elements including ECM, cancer-associated fibroblasts (CAFs), and cancer cells become more visible, which causes tumors to expand quickly in size and stiffen. One of the few readily observable mechanical characteristics of tumors that helps doctors anticipate malignancy and prognosis is the fast increase in stiffness [97]. Internally produced pressures enable the tumor to displace nearby healthy tissue and invade neighboring regions as it grows and becomes stiffer. Thus, these forces created within the tumor and those brought on by interactions with its surroundings directly aid tumor growth. Tumor cells experience both fluid and solid stress as a result of these mechanical forces [98].

Typically, the non-fluid elements of the tumor produce solid tension. The discovery that blood and lymphatic arteries are mechanically squeezed in the course of tumor development provided the first support for the presence of solid stress within tumors. In tumors, growth-induced solid stress builds up when the cancer cells multiply quickly [99]. Cells multiply quickly during this phase, placing strain on the tumor's microenvironment and ultimately the adjacent healthy tissues. Additionally, to the solid stress produced by the tumor itself, the adjacent tissue's efforts to thwart tumor growth also cause external solid stress [100]. In a nutshell, solid stressors have a direct impact on the evolution of tumors in two ways. First, they impart direct mechanical stress to tumor cells, altering their genetic expression and causing them to become more malignant and invasive. Second, solid stress distorts lymphatic and blood arteries to cause hypoxia [101]. As the name implies, fluid stresses are caused by forces that the fluid components of the tumor produce. This comprises

the shear forces brought on by capillaries, interstitial fluid movement, microvasculature, and blood and lymphatic flow inside the vessels. The shrinkage of blood/lymphatic arteries by solid stress has a significant impact on the fluid stress placed on the surrounding epithelial tissue, proving that these stresses are in fact intricately linked [102]. Vascular constriction increases the vessel's resistance to lymphatic flow by narrowing its cross-sectional area, which also raises shear stress, increases interstitial fluid volume, and lowers perfusion rates. The capacity of lymphatic arteries to remove extra fluid from the tumor is severely restricted by this decline in perfusion rates and flow, which eventually raises interstitial fluid pressure in nearby tumor tissue. Additionally, the efficiency of chemotherapy and immunotherapies is significantly harmed by the constriction of blood and lymphatic arteries [103].

Cancer cells are in a completely different physiological environment within tumors due to elevated solid and fluid stress. Mechanically acting strain and compression on the cells triggers pathways that lead to tumor formation, boosts cell proliferation, and encourages mass migration [104, 105]. In addition to having more stiffness, cancer cells also create more force than surrounding tissues, making them more vulnerable to it. While measuring the solid stress within tumors has shown to be significantly more difficult than measuring the bulk stiffness of tumors, this problem is not insurmountable [106]. Individual tumor cells are now being measured for solid stress by researchers. Recently, Nia et al. presented the experimental methodology for in-situ 2D mapping of solid stress [107].

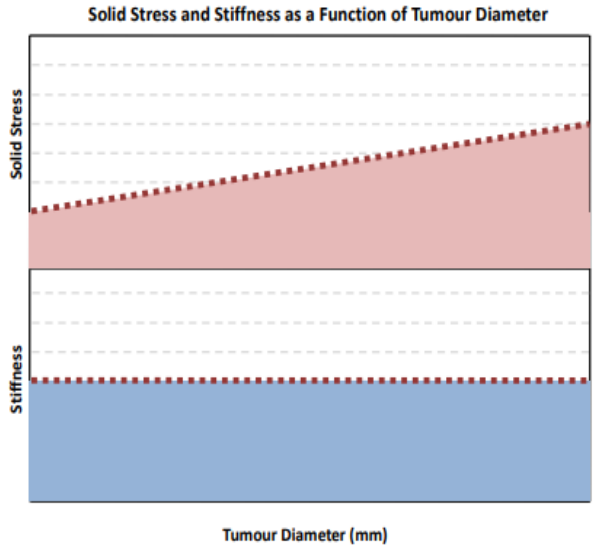

Fig. 6. How solid stress and stiffness change with tumour diameter. Greater solid stress within the tumour is related to increased tumour diameter since the ECM's stiffness remains constant [1].

Investigators use preset geometry to encase the tumor in agarose gel and record distortion after making a small hole to achieve this mapping by carefully releasing the solid tension in tissues [108]. Using both mathematical modelling and experimental investigation, the following key discoveries were made as shown in Figure 6: the stiffness remains constant, solid stress grows linearly with tumor size, and neighboring tissue of healthy nature considerably adds to the solid stress within the tumor. The results imply that the tumor's stiffness is independent of the solid stress applied to the tumor cells [1].

3. CONCLUSIONS

This review addresses the intricate and complex role of the ECM in tissue-genesis and cancer progression. Over the past 20 years, research has shown how critical the ECM is in controlling key physiological processes such as determination of stem cell lineage, cell migration, and its propagation. Consequently, perspectives on cancer have shifted to view it as a disease marked by both uncontrolled cell development and microenvironment instability. Throughout all stages of cancer growth, the apparently static extracellular matrix undergoes dynamic remodeling due to complex interactions among cancer cells, resident cells, and acellular components. Our understanding of the role of extracellular matrix (ECM) in cancer development has advanced, highlight possible therapy targets for lowering the propensity of cancer to spread. However, the temporal sensitivity and specificity required to successfully slow down the propagation of the tumor cells are revealed by the failure to efficiently target wide protein ranges, such as MMPs and collagen.

Neoplastic cells in tumors endure increased mechanical stress when they multiply quickly, which mechanically stimulates tumorigenic pathways, promotes migration, and causes hypoxia. Investigating the correlation between mechanical stress in tumors and their detrimental behavior as well as angiogenesis is crucial while doing cancer

research. These signaling pathways that link external mechanical stress to malignant behavior provide excellent therapeutic targets to halt the spread of cancer. Understanding the link between elevated solid stress and angiogenesis pathways will also provide light on potential improvements in medication delivery.

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5. CONFLICT OF INTEREST

The authors declared to no conflict of interest.

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Research Article

A Study of Egg Quality, Fertility and Hatchability in Kashmiri Rhode Island Red Chicken Breed

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Abstract: The fertility and hatchability of the eggs are traits of economic importance in poultry production. The Kashmiri Rhode Island Red (RIR) breed maintained under an intensive management system by the Government Poultry Farm Chacksagher, Mirpur, was assessed to study productive performance. The birds were provided with a standard formulated chick mash throughout the brooding period, grower ration for an additional period and layers mash from 21 weeks onwards. The results revealed that up to week 40, Kashmiri RIR males and females had an average weight of 2.74 ± 0.05 kg and 2.02 ± 0.04 kg, respectively. The mean age at sexual maturity was found to be 148 ± 1.22 days. The overall egg production percentage was 72.21 ± 2.40 with mean egg weight 52.94 g, shape index 77.90%, shell weight 4.94 g, shell thickness 0.30 mm, albumen weight 28.88 g, and yolk weight 16.26 g. The albumen and yolk height were 6.51 and 16.26 mm, respectively. The fertility was 88.60% while hatchability was 93.48% based on fertile eggs. Significant positive correlations were observed between age/fertility (0.712), age/hatchability of fertile eggs (0.561), age/hatchability of all eggs (0.681), fertility/hatchability of fertile eggs (0.857), fertility/hatchability of all eggs (0.982) hatchability of fertile eggs/hatchability of all eggs (0.938). In contrast significant negative correlations were assessed between traits like age/dead in germ (-0.748), shape index/dead in shell (-0.798), dead in germ/fertility (-0.748), dead in germ/hatchability of fertile eggs (-0.505) and dead in germ/hatchability of all eggs (-0.540). This study concluded that the Kashmiri RIR breed is an improved dual-purpose exotic breed which performs exceptionally well under intensive management systems in AJK, indicating its suitability for sustainable rural poultry production. The results provide a foundation for further genetic improvement programs and emphasize the importance of tailored management practices for optimizing productivity.

Keywords: Egg Quality, Fertility, Hatchability, Kashmiri Rhode Island Red, Chicken.

1. INTRODUCTION

Animal protein sources for the human population have been a significant concern in Azad Jammu and Kashmir (AJK), with reports indicating that many people are suffering from protein deficiency [1]. The World Health Organization (WHO) has estimated that the average daily requirement of animal protein is 27 grams per person. However, in Pakistan, this figure is significantly lower, with an average intake of only 17 grams per person. Notably, only 5 grams of these 17 grams of animal protein comes from poultry sources [2, 3]. Animal protein is a vital macronutrient that not only provides a complete profile of essential amino acids but also contains bioactive compounds critical for various physiological functions. Inadequate consumption of animal protein increases the risk of stunting, compromised immune systems, and impaired cognitive development, particularly in vulnerable populations such as children [4].

To address this issue, commercial chicken farming plays a critical role in meeting the protein demands of the region. The poultry sector in AJK has increasingly focused on exotic breeds due to

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their higher egg and meat production. These exotic breeds, along with their crossbreeds, are not only raised by farms but also by rural farmers. They are particularly popular in small households across rural, urban, and semi-urban areas of AJK due to their higher productivity compared to indigenous chicken breeds [5].

However, one of the major challenges in promoting commercial poultry farming in rural regions is the low adaptability of imported chicken breeds to the local climatic conditions. The exotic breeds need to be further studied to assess their full genetic potential and suitability for the region's environmental factors [6].

Previous studies have shown significant differences in the productive and reproductive performance of different chicken breeds. Exotic breeds, in particular, tend to show higher growth rates [7-9]. Fertility and hatchability are two critical aspects of reproductive performance, which are influenced by both genetic and environmental factors [10, 11]. Egg quality is another crucial factor affecting fertility and hatchability. Egg quality can be divided into external features, such as shell strength, and internal components like albumen height and yolk quality [12-14]. These traits vary between breeds and are vital for both consumer preference and the economic success of poultry farmers [15].

The poultry industry in Azad Jammu and Kashmir (AJK) has long been a neglected subsector within agriculture, facing challenges in meat and egg production, which result in lower returns and limited investment. The introduction of the Kashmiri Rhode Island Red (RIR) chicken breed, a dual-purpose exotic breed, could offer potential solutions to these persistent challenges. The Kashmiri RIR breed, introduced to AJK in 1975, has undergone nearly 200 generations of local production, resulting in a breed uniquely acclimatized to the region's environmental and management conditions. This breed is valued for its dual-purpose utility—providing both meat and eggs—as well as its adaptability to local rural poultry systems.

The adaptability of exotic breeds like RIR to local climatic conditions is essential for realizing their full genetic potential. Environmental factors such as temperature, humidity, and high-altitude terrain can significantly influence productivity and reproduction [16]. The Kashmiri RIR breed, however, represents a valuable genetic resource for rural communities in AJK due to its resilience, adaptability, and economic feasibility.

This study aims to systematically evaluate the egg quality, fertility, and hatchability of the Kashmiri RIR chicken, contributing to a deeper understanding of the breed's role in improving rural livelihoods and addressing regional protein deficiencies. The primary objective is to investigate the breed's acclimatization to local environmental conditions in AJK after generations of controlled breeding. Specific goals include: (i) assessing the external and internal egg quality characteristics, including weight, shell thickness, albumen height, and yolk color, were analyzed to determine their impact on hatchability and fertility, (ii) evaluating reproductive Performance to understand how effectively the breed reproduces under intensive management conditions; and (iii) exploring genetic and environmental interactions to determine how well the breed has adapted to AJK's climatic and management conditions.

By introducing the Kashmiri RIR breed, this research aims to stimulate increased interest, investment, and transformative change within the poultry sector in AJK. The findings are expected to provide actionable insights that will help optimize management practices, formulate strategies for sustainable poultry production, and ultimately address both economic and nutritional challenges in the region.

2. MATERIALS AND METHODS

2.1. Study Area

To study the productive and reproductive performance of RIR chicken along with the egg traits, data was collected from the Government Poultry Farm Chacksagher, Mirpur, located between latitude 33° 8' 54.2112'' N and longitude 73° 45' 6.3720'' E, lies at the foothills of the Himalayas mountain range of an altitude of 648 meters or 2,126 feet above sea level [17]. Mirpur has a humid subtropical climate. The average annual temperature is 25.1 °C. The average annual rainfall is 1,380 millimeters or 54.3 inches [18]. The

farm covers an area of approximately 5 acres and has a capacity of housing 1,500 birds at a time. It is equipped with separate housing units for different age groups, semi-automated feeders, drinkers, and environmental control systems to maintain optimal temperature and humidity. The farm provides a controlled environment conducive to scientific research on poultry breeds.

2.2. Intensive Housing System

The 1024 birds of the Kashmiri RIR breed were kept at a comfortable temperature and humidity level with sufficient space and adequate ventilation. Straw was spread out on the floor to serve as an absorbent for the faecal droppings. Newly hatched chicks were nurtured in an electrically heated brooder for three weeks. The male and female ratio was kept at 1:10 at sexual maturity. Birds were provided with a standard formulated chick mash throughout the brooding period. During the growing phase, a grower ration was used, followed by layers mash from 21 weeks onward. This systematic feeding ensured nutritional adequacy for optimal growth and production.

2.3. Study Design

2.3.1. Body weight

Body weight (in kilograms) for both sexes was recorded weekly from one week to 40 weeks of age using a digital spring balance. The mean body weight was then calculated for each measurement. The body weight gain was determined using the following formula [9]:

$$\text{Gain in body weight} = \text{Final body weight} - \text{Initial body weight}$$

2.3.2. Egg production traits and sexual maturity

Age and body weight at sexual maturity, number of live hens per day and number of eggs on the daily basis was recorded during the 60 weeks' trial. The eggs were collected twice daily, in the morning and evening and the egg number was counted. Age and body weight at sexual maturity were determined based on the appearance of the first egg, which was monitored daily until 50% of the flock started laying eggs. This point was considered the age of sexual maturity. Birds were weighed immediately upon the onset of egg production to record body weight at this stage. The egg production percentage was calculated using a formula (adopted from Khawaja *et al.* [9];

$$Egg\ production(\%) = \frac{Total\ no.of\ eggs}{Live\ hens\ per\ day} \times 100$$

2.3.3. External egg quality traits

A total of 20 eggs were chosen from each poultry shed and evaluated for external and internal quality traits. The external egg characteristics; egg weight (grams): by using a digital spring balance; egg length and width (millimeters): by using vernier calliper with the least count of 0.05 mm; shell weight (g) and thickness (mm): which was measured at three different points and the average of the three was taken; eggshell colour was monitored by visual comparison with an eggshell colour fan, a series of graded (1-15) standard colorimetric system; and egg shape index (SI) which is defined as ratio of the egg width to egg length was used to categorized egg shape. SI was calculated as described by Kumar *et al.* [19]:

$$Shape\ index\ (\%) = \frac{Egg\ width}{Egg\ length} \times 100$$

2.3.4. Internal egg quality traits

For the determination of internal egg quality, various traits were evaluated including albumen weight (g), albumen height (mm), yolk weight (g), yolk height (mm), yolk color and Haugh Unit. These traits are critical indicators of egg quality because they influence the egg freshness, nutritional value, hatchability and consumer preferences.

2.3.4.1. Albumen and yolk height:

Each egg was broken out onto a flat surface and then allowed to sit for five minutes. For the measurement of albumen and yolk height, a height/depth gauge with the least count of 0.01 mm was used.

2.3.4.2. Albumen and yolk weight:

After measuring the height of albumen and yolk, they were detached carefully and weighed separately using digital electronic balance.

2.3.4.3. Yolk color:

A Roche color fan, standard colorimetric scheme ranged 1-15, 1 being pale yellow and 15 being deep vivid reddish orange was used to record the yolk color of eggs. The fan was used by visually comparing the yolk color under natural light to the standardized color chart. The score was recorded by two trained observers to ensure consistency and minimize subjective bias.

2.3.4.4. Haugh unit (HU):

The Haugh unit (HU) was calculated by using two egg parameters namely, height of albumen and egg weight using the following formula [20]:

$$HU = 100 \log(AH + 7.6 - 1.7\, EW^{0.37})$$

Where, HU = Haugh Unit,
AH = Observed albumen height (mm), and
EW = egg weight (g)

2.3.5. Fertility and hatchability

The eggs were collected from the birds' sheds daily and transported for sorting out, excluding cracked, dirty or distorted. The eggs were then transported to the hatchery and stored at 16 °C with a relative humidity of 70–80% for the evaluation of fertility, hatchability, dead in shell, and dead germ percentage [21]. Eggs were automatically incubated in accordance with conventional temperature and humidity settings that were automatically checked [22] with automatic turning of eggs through 90° in the incubator after every two hours. Individual eggs were checked through candling on the 5th and 18th day of incubation to provide a precise evaluation of the embryo's developmental stage. Eggs without signs of embryo development were counted and removed to determine fertility percentage, as outlined by Khan *et al.* [23]. The rest of the eggs having live embryos were then shifted to the hatching chamber of the incubator. Hatching process started on 19th day and ended at 21st day, the chicks were removed and counted. The fertility is the proportion of the egg that develop viable embryo upon incubation and the hatchability is the percentage of the fertile egg that successfully hatch into chicks. The fertility (%) and hatchability (%) was calculated as described by Ahmedin and Mangistu [24]:

$$Fertility\ (\%) = \frac{Total\ fertile\ eggs}{Total\ eggs\ set} \times 100$$

$$Hatchability\ \%\ (fertile\ eggs) = \frac{Chicks\ hatched}{Total\ fertile\ eggs} \times 100$$

$$Hatchability\ \%\ (total\ eggs\ set) = \frac{Chicks\ hatched}{Total\ eggs\ set} \times 100$$

2.3.6. Statistical analysis

The data was expressed as mean ± Standard Error of Mean (SEM) and coefficient of variance (CV). The body weight, egg production traits, and egg quality parameters (both external and internal) were presented as mean ± SEM to summarize central tendency and variability. The CV was calculated for external traits such as egg weight, shell thickness, and shape index, as well as internal traits like albumen height, yolk weight, and Haugh unit, to assess the relative variability of these measurements. The fertility and hatchability of eggs were studied in percentages. The relationships between various egg quality traits and reproductive traits were analyzed using GraphPad Prism 6.01 software. Pearson's correlation coefficient was applied to measure the strength and direction of linear relationships between traits. Statistical significance will be declared at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Body Weight

The live body weight of Kashmiri RIR breed from week 1 to 40 along with average weight gain is presented in Table 1. In this study Kashmiri RIR chickens recorded a drastic increase in body weight up to week 25. From week 30, the body weight of RIR increases gradually (mainly in females) and remains noticeably constant for some weeks. This steadiness in body weight can be attributed to the management of an intensive housing system. Moreover, the birds were fed a controlled amount of feed by their age. The amount of feed was increased until week 21. After that the same amount of feed (114 g/bird) was provided to the chickens which could have been the reason for the control in their body weights. The fluctuations in body weight of both sexes concerning age are presented in Figure 1.

In contrast to the results of the current study, Rahman *et al.* [25] in Bangladesh reported Lower body weight for RIR being 1485.22 g at 24 weeks

Table 1. Mean ± SEM for body weight of Kashmiri RIR breed up to 40 weeks.

Age (weeks)	Body weight of males (kg)	Gain in body weight (males)	Body weight of females (kg)	Gain in body weight (females)
1	0.03 ± 0.001		0.03 ± 0.001	
2	0.05 ± 0.001	0.02 ± 0.001	0.05 ± 0.001	0.02 ± 0.001
3	0.10 ± 0.002	0.05 ± 0.002	0.10 ± 0.002	0.05 ± 0.002
4	0.17 ± 0.003	0.07 ± 0.003	0.17 ± 0.003	0.07 ± 0.003
5	0.24 ± 0.005	0.08 ± 0.006	0.22 ± 0.005	0.11 ± 0.001
6	0.33 ± 0.01	0.09 ± 0.01	0.28 ± 0.01	0.04 ± 0.009
7	0.42 ± 0.01	0.17 ± 0.03	0.36 ± 0.01	0.14 ± 0.03
8	0.52 ± 0.02	0.09 ± 0.02	0.43 ± 0.02	0.07 ± 0.02
9	0.74 ± 0.04	0.17 ± 0.01	0.55 ± 0.05	0.10 ± 0.04
10	0.89 ± 0.05	0.15 ± 0.06	0.71 ± 0.03	0.17 ± 0.04
11	0.89 ± 0.08	0.03 ± 0.13	0.79 ± 0.06	0.02 ± 0.09
12	1.12 ± 0.03	0.67 ± 0.15	0.87 ± 0.05	0.47 ± 0.16
13	1.30 ± 0.04	0.19 ± 0.05	0.96 ± 0.04	0.09 ± 0.04
14	1.51 ± 0.06	0.21 ± 0.06	1.01 ± 0.02	0.04 ± 0.03
15	1.57 ± 0.05	0.06 ± 0.06	1.15 ± 0.03	0.15 ± 0.03
16	1.68 ± 0.04	0.13 ± 0.07	1.24 ± 0.03	0.08 ± 0.05
17	1.88 ± 0.04	0.07 ± 0.10	1.38 ± 0.03	0.08 ± 0.06
18	2.06 ± 0.05	0.30 ± 0.06	1.51 ± 0.02	0.20 ± 0.04
19	2.06 ± 0.05	0.007 ± 0.02	1.55 ± 0.03	0.03 ± 0.02
20	2.06 ± 0.06	0.002 ± 0.03	1.56 ± 0.04	0.009 ± 0.02
21	2.20 ± 0.04	0.14 ± 0.05	1.66 ± 0.06	0.10 ± 0.08
22	2.20 ± 0.05	0.0005 ± 0.05	1.67 ± 0.05	0.009 ± 0.07
23	2.21 ± 0.09	0.007 ± 0.09	1.87 ± 0.06	0.20 ± 0.05
24	2.24 ± 0.06	0.03 ± 0.10	1.89 ± 0.09	0.02 ± 0.06
25	2.30 ± 0.07	0.06 ± 0.04	1.89 ± 0.04	0.003 ± 0.06
26	2.36 ± 0.08	0.06 ± 0.009	1.89 ± 0.05	0.005 ± 0.01
27	2.43 ± 0.10	0.07 ± 0.04	1.89 ± 0.05	0.0005 ± 0.05
28	2.43 ± 0.09	0.002 ± 0.10	1.92 ± 0.10	0.03 ± 0.09
29	2.46 ± 0.07	0.03 ± 0.09	1.92 ± 0.06	0.002 ± 0.12
30	2.52 ± 0.07	0.06 ± 0.03	1.92 ± 0.06	0.0005 ± 0.03
31	2.53 ± 0.06	0.009 ± 0.03	1.93 ± 0.07	0.009 ± 0.06
32	2.55 ± 0.05	0.02 ± 0.03	1.95 ± 0.11	0.02 ± 0.06
33	2.60 ± 0.06	0.05 ± 0.03	1.95 ± 0.06	0.001 ± 0.06
34	2.66 ± 0.08	0.05 ± 0.03	1.97 ± 0.04	0.01 ± 0.06
35	2.66 ± 0.07	0.004 ± 0.09	1.97 ± 0.06	0.005 ± 0.03
36	2.66 ± 0.07	0.004 ± 0.08	1.97 ± 0.08	0.001 ± 0.03
37	2.67 ± 0.08	0.01 ± 0.11	1.96 ± 0.05	-0.006 ± 0.09
38	2.66 ± 0.09	-0.002 ± 0.07	2.01 ± 0.09	0.05 ± 0.11
39	2.67 ± 0.11	0.007 ± 0.15	2.02 ± 0.05	0.004 ± 0.11
40	2.74 ± 0.05	0.07 ± 0.13	2.02 ± 0.04	0.002 ± 0.06

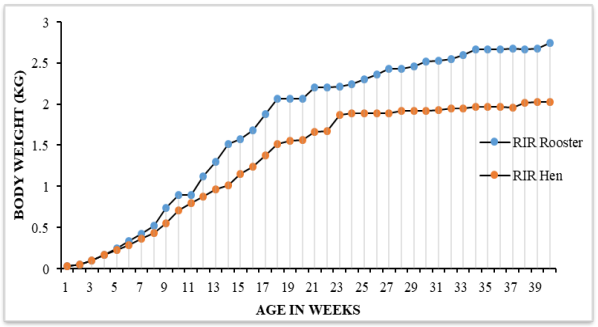

Fig. 1. Relationship between age and body weight in Kashmiri RIR breed.

of age and 1384.44 g at 40 weeks of age. Khawaja *et al.* [8] in Pakistan also recorded lower final body weight (1640 g), body weight gain (1608 g) in RIR up to 20 weeks. Weight gain in RIR chicken during the first 20 weeks was 1257.76 ± 4.52 in Rawalpindi, Pakistan [26]. Nowier *et al.* [27] showed that RIR in Egypt recorded body weight of 2207.25 ± 98.82, 2224.00 ± 66.89 and 2299.73 ± 70.7 g at 34th, 38th and 42nd week of age, which is lower as compared to the figures obtained in the present study.

It was discovered that poultry production varies from farm to farm; it is influenced by several factors, namely, the number of birds on the farm, the mortality rate, the quality and quantity of feed consumed, the temperature range, seasonal fluctuations, and the ratio of pure breeds [9]. Gholami *et al.* [28] obtained the highest production for broiler chickens in an alpine climate. The climate of AJK is warm in summer and cold in winter and can be classified as a subtropical highland type [18]. The current study's findings may lead to the conclusion that better production performance was recorded for RIR in summer and winter in the subtropical climate.

3.2. Egg Production Traits and Sexual Maturity

Egg production is the yield of a bird's total performance regarding factors like egg number, rate of lay, age at sexual maturity, and characteristics of the eggs' quality [27]. The egg production and quality traits in Kashmiri RIR breed are summarized in Table 2. The mean age at sexual maturity of RIR was found to be 148 ± 1.22 days in the current study. Similarly, Khawaja *et al.* [9] reported that for the RIR breed the age at sexual maturity is 147 ± 1.15 days. The mean age at sexual maturity, 50% egg production and peak egg production were summarized to be 252.5, 273.3 and 294.5 days, respectively, in Bangladesh [29]. Maturity occurs at a specific age and body weight and is influenced by a variety of factors such as nutrition, temperature, light intensity, and many others [9]. The average body weight at sexual maturity for Kashmiri RIR hens was calculated to be 1.69 ± 0.03 kg. In contrast, lower body weight of RIR at sexual maturity (1296.3 g) and peak egg production stages (1538.8 g) was reported in Bangladesh [29]. In Rawalpindi RIR, Khawaja *et al.* [9] recorded 41% egg production in RIR breed which is much lower than that of present study (72.21 ± 2.40%; Table 2).

3.3. External Egg Quality Traits

3.3.1. Egg weight

The mean egg weight of Kashmiri RIR in the present study was 52.94 ± 0.35 g (Table 2). Similar value of mean egg weight for RIR was reported in Pakistan by Ashraf *et al.* [6] being 53.10 ± 0.30 g and Farooq *et al.* [12] being 53.94 ± 0.69 g while higher mean egg weight was presented in other studies conducted in Ethiopia (55.56 ± 1.79 g) [19], Egypt (56.29 ± 0.99 g) [27], and Slovak Republic (57.60 ± 0.76 g) [30], for RIR breed. In contrast, lower mean egg weight was reported for RIR being 49.07 ± 0.60 g under intensive management in Pakistan [31]. Amao [32] also indicated low average egg weight for the RIR genotype i.e., 48.02 g. The difference in egg weights between different studies could be due to many factors such as age, management, egg production level, and agroecological conditions.

3.3.2. Egg length and width

The mean values of egg length and width of Kashmiri RIR hens recorded in this study were 5.37 ± 0.02 cm and 4.18 ± 0.009 cm, respectively. The findings of this study are consistent with Farooq *et al.* [12] who recorded the mean egg length and width for RIR as 5.57 cm and 4.19 cm, respectively, in Pakistan. Hanusova *et al.* [30] in Slovak Republic summarized almost similar figures of egg length (5.62 ± 0.03 cm) and egg width (4.21 ± 0.02 cm) for RIR. The range of egg length (5.65 ± 0.17 cm) and width (4.38 ± 0.11 cm) for eggs of RIR reported by Kumar *et al.* [19] in Ethiopia was higher. However, lower mean values were recorded for egg length and width in RIR kept under intensive management in Pakistan [31].

Table 2. Mean ± SEM of egg production and quality traits in Kashmiri RIR breed.

Traits	Mean ± SE
Age at sexual maturity (days)	148 ± 1.22
Average body weight at sexual maturity (kg)	1.69 ± 0.03
Egg production (%)	72.21 ± 2.40
External egg quality traits	
Egg weight (g)	52.94 ± 0.35
Egg length (cm)	5.38 ± 0.02
Egg width (cm)	4.18 ± 0.009
Eggshell thickness (mm)	0.30 ± 0.003
Eggshell weight (g)	4.94 ± 0.03
Eggshell color	9.23 ± 0.17
Shape index (%)	77.90 ± 0.20
Internal egg quality traits	
Albumen height (mm)	6.51 ± 0.08
Yolk height (mm)	15.28 ± 0.16
Albumen weight (g)	28.88 ± 0.21
Yolk weight (g)	16.26 ± 0.11
Yolk color	7.232 ± 0.08
Haugh unit	82.57 ± 0.53

3.3.3. Egg shell quality

The quality of eggshell is determined by weight, thickness, percentage and strength. Eggshell quality varies depending upon environmental factors, feed quality, and chicken genotype [27]. In the present study, mean eggshell thickness was found out to be 0.30 ± 0.003 mm (Table 2). The findings of the present study are consistent with the results obtained by Khawaja *et al.* [9] who reported the eggs of RIR breed with eggshell thickness of 0.29 ± 0.13 mm. Higher eggshell thickness in RIR was reported in Pakistan by Farooq *et al.* [12] (0.39 ± 0.01 mm), Ashraf *et al.* [6] (0.36 ± 0.004 mm) and Nowier *et al.* [27] (0.41 ± 0.04 mm). The lower values of eggshell thickness in the present study could be attributed to the lack of nutrients in the diet as well as fluctuations in the environmental conditions. Eggshell thickness should be between 0.33 and 0.35 mm for the best hatchability [33]. Poor or thin shelled eggs have less chance of hatching. Ahmed *et al.* [34] reported that thick shelled eggs have a higher percentage hatchability (78.85%) than thin-shelled eggs (67.76%).

The mean eggshell weight (4.94 ± 0.03 g) documented for Kashmiri RIR (Table 2) was lower than that observed for RIR being 5.45 ± 0.12 g in Egypt [27], and 6.21 ± 0.06 g in Slovak Republic [30]. In contrast, Farooq *et al.* [12] in Peshawar, Pakistan presented lower mean eggshell weight for RIR (4.77 ± 0.09 g). The mean eggshell colour of the Kashmiri RIR breed presented in this study was 9.23 ± 0.17. The eggshell colour recorded in this study varied between the ranges of 5-14.

3.3.4. Shape index

Eggs have different shapes which can be differentiated using the shape index. The most often encountered egg shapes are sharp, normal (standard) and round, which were labelled on the shape index (SI) scale as <72, 72-76, and >76, respectively [35]. In the present study, the shape index of eggs was 77.90 ± 0.20 which was higher than 76 on the SI scale, hence the eggs are categorized as round. Nowier *et al.* [27] in Egypt and Kumar *et al.* [19] in Ethiopia also reported round shapes for RIR eggs with 76.74 and 78.43 egg shape index, respectively. In contrast, Ali and Anjum [31] recorded a standard shape with SI equal to 73.08 for RIR under the intensive system in Pakistan.

3.4. Internal Egg Quality Traits

3.4.1. Albumen and yolk height

Mean albumen and yolk height in Kashmiri RIR was found to be 6.51 ± 0.08 mm and 16.26 ± 0.11 mm (Table 2). The results compiled by Hanusova *et al.* [30] support the findings of the present study who reported RIR eggs in temperate regions to have yolk height of 16.97 ± 0.43 mm. Higher mean albumen and yolk height in RIR being 7.87 ± 0.65 mm and 17.34 ± 0.76 mm was reported in Ethiopia [19]. Khawaja *et al.* [9] proposed that the RIR breed produces eggs with average albumen height of 0.90 ± 0.07 cm in Pakistan. High thermal stress negatively affects albumen height and yolk leading to lower values of albumen and yolk height, hence ultimately Haugh unit decreases [36].

3.4.2. Albumen and yolk weight

The mean albumen and yolk weights recorded in this study were 28.88 ± 0.21 g and 16.26 ± 0.11 g, respectively. In Rawalpindi, Pakistan the RIR breed

produced eggs with higher albumen (32 ± 1.15 g) and yolk (20 ± 0.28 g) weight as compared to that of the present study [9]. The RIR breed in central Europe with temperate climate was documented to produce eggs with heavier albumen (32.78 ± 0.73 g) and yolk (18.61 ± 0.20 g) as compared to the Kashmiri RIR breed of the present study found in subtropical climate conditions [30]. Nowier *et al.* [27] recorded higher values of albumen (34.95 g) and yolk weight (18.8 g) in Egypt. In contrast, Islam and Dutta [37] reported lower yolk weight (11.20 g) but heavier albumen weight (36.10 ± 4.46 g) in tropics.

3.4.3. Yolk colour

The most important aspect in any consumer survey pertaining to egg quality is the yolk colour [38]. Mean yolk colour of RIR was found to be 7.23 ± 0.08 according to Roche colour fan (Table 2). Kumar *et al.* [18] reported higher mean yolk colour being 9.25 ± 2.75 for RIR. In temperate regions, RIR eggs were observed to have darker yolk (11.10 ± 0.20) as documented by Hanusova *et al.* [30]. Supplemented maize makes a significant contribution to improved yolk colour intensity among feed ingredients. As a result, if a hen has access to green grass or supplemented feed ingredients containing carotenoids or xanthophylls, it will be sufficient to give the yolk the desired colour [39].

3.4.4. Haugh unit (HU)

Good quality eggs have a higher value of haugh units [40]. The haugh unit value for Kashmiri RIR was figured out 82.57 ± 0.53 which was almost consistent with the HU values recorded for RIR being 83.67 ± 3.78 in Egypt [19]. Some studies in UK have shown that for eggs with haugh unit below 60 there is a consumer resistance [41]. The results of this study were in contrast with the higher HU values reported in many previous studies in Pakistan and Egypt being 102.57 ± 0.59 and 87.96 ± 0.70, respectively [6, 27]. As compared to our findings, Monira *et al.* [13] discovered that fresh RIR eggs have a lower haugh unit.

3.5. Correlation among Various Traits for Kashmiri RIR Breed

The correlation assessment between various production and egg quality traits for Kashmiri RIR breed is presented in Table 3. The total of 105 correlations (between all combinations) were assessed, 56 were found positive and 38 were negative and 11 show no correlation. The significant positive correlations were found between Age/body weight (BW 0.83, P = 0.0001), Age/Yolk weight (0.74, P = 0.02), Egg weight/egg length (0.73, P = 0.02), Egg weight/egg width (0.72, P = 0.02), Egg weight/shell weight (0.88, P = 0.001) and Egg length/shell weight (0.83, P = 0.004) and Egg width/shell weight (0.71, P = 0.02).

Hailemariam *et al.* [42] reported that egg weight correlates positively and significantly with egg length (0.987), egg width (0.984) and eggshell weight (ESW; 0.964) for different chicken breeds in Ethiopia. The results showed that egg weight positively influences external quality traits in Kashmiri RIR hens. Non-significant correlations of EW with ESW were reported for RIR in tropics [37].

Non-significant positive correlations of egg weight and body weight (0.42) were in accordance with the results documented by Barua *et al.* [29] in RIR and Dana *et al.* [43] in Horro chicken of Ethiopia. Given that both criteria were found to be positively correlated in the current study, the production of eggs with higher egg weights in the Kashmiri RIR breed can also be attributable to increased body weight.

Verma *et al.* [44] assessed significant positive correlations ($P<0.01$) between egg shape index and egg weight in Aseel and Kadaknath hens which conflicts with the nonsignificant negative correlation of shape index and egg weight of current findings. In contrast with the findings of the present study, Farooq *et al.* [12] and Zita *et al.* [45] mentioned non-significant and positive correlations between egg weight and eggshell thickness.

The positive correlations of egg weight with internal egg quality traits such as albumen and yolk weight reported by Khawaja *et al.* [46] are in accordance with the results of the current study (0.42, 0.30, respectively). The results showed that larger eggs would have heavier albumens and yolks. Positive correlations of EW with albumin weight (AW; 0.96) and yolk weight (YW; 0.72) were also reported in the tropics [37]. Hailemariam *et al.* [42] also summarized similar results for correlations of

Table 3. Correlation among various productive and reproductive traits of Kashmiri RIR breed.

	Age	EPP	BW	EW	EL	EWD	ESW	EST	ESC	SI	AW	AH	YW	YH	YC	HU
Age	1															
EPP	-0.061	1														
BW	0.834***	0.326	1													
EW	0.361	0.769	0.422	1												
EL	0.327	-0.25	0.121	0.735**	1											
EWD	0.132	-0.23	-0.110	0.725**	0.548	1										
ESW	-0.153	0.090	-0.256	0.887***	0.840**	0.719*	1									
EST	0.374	-0.26	0.134	-0.089	-0.101	0.380	-0.10	1								
ESC	-0.263	-0.07	-0.71**	0.193	-0.032	0.334	0.258	0.363	1							
SI	-0.209	0.044	-0.205	-0.107	-0.547	0.398	-0.21	0.518	0.377	1						
AW	-0.303	0.006	-0.575	0.427	0.252	0.561	0.557	0.032	0.806	0.287	1					
AH	-0.465	0.472	0.048	0.680	0.761	0.119	0.877	-0.62	-0.56	-0.95	-0.77	1				
YW	0.748**	-0.61	0.395	0.302	0.406	0.409	0.096	0.562	-0.20	-0.03	-0.26	0.280	1			
YH	0.005	-0.35	-0.947	0.019	-0.248	0.007	-0.09	0.790	0.921	0.291	0.525	-0.20	-0.12	1		
YC	-0.338	0.123	-0.385	0.385	0.168	0.506	0.495	-0.32	0.249	0.294	0.561	-0.68	-0.45	0.848	1	
HU	-0.839	0.892	0.375	0.138	0.292	-0.446	0.446	-0.42	-0.65	-0.63	-0.98**	0.819	-0.27	-0.35	-0.761	1

*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001

BW = Body weight (g), EPP = Egg production percentage, EW = Egg weight (g), EL = Egg Length (cm), EWD = Egg width (cm), ESW = Eggshell weight (g), EST = Eggshell thickness (mm), ESC = Eggshell color, SI = Shape Index (%), AW = Albumen weight (g), AH = Albumen height (mm), YW = Yolk weight (g), YH = Yolk height (mm), YC = Yolk color, HU = Haugh Unit.

EW with albumen (0.891) and yolk (0.657) weight. The negative correlation between albumen height and albumen weight (-0.20) found in this study was contradictory to the results of Begli *et al.* [47] who reported high positive correlations of albumen height with albumen weight (0.52). Khawaja *et al.* [46] also assessed the positive and significant correlation of albumen height/albumen weight (0.768) and albumen height/yolk weight (0.699), non-significant positive ($P < 0.05$) correlations between albumin height and shell thickness (0.019).

In the present study, positive but non-significant correlations were observed between albumen height and egg weight (0.67) while Scott and Silversides [48] reported significant positive correlations between these two quality traits. Khawaja *et al.* [46] also mentioned significant and positive correlations between albumen height and egg weight (0.772, $P < 0.01$). The significant negative correlations were found between BW/shell colour (-0.71, $P = 0.03$) and albumen weight/HU (-0.98, $P = 0.01$). In the present study, it was observed that there is a negative non-significant correlation (-0.60) of age with egg production. This implies that as the age of the birds increases, the egg production percentage decreases as presented in Figure 2. These findings are consistent with those of Joyner *et al.* [49].

3.6. Fertility and Hatchability

During the four months period, from January to April, 22600 eggs of RIR breed were selected to evaluate the fertility and hatchability percentages.

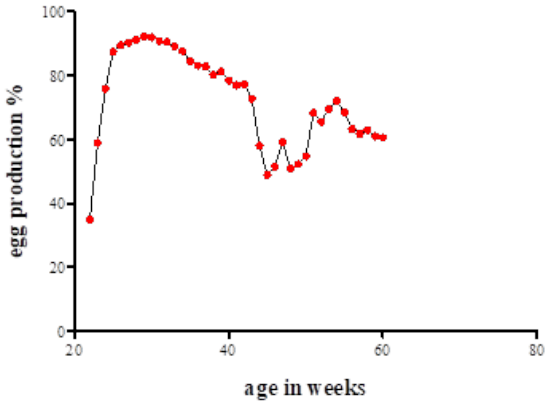

Fig. 2. Correlation between age and egg production in Kashmiri RIR.

Among the total, 2584 eggs were considered unsuitable and removed due to abnormalities, very small size, or poor quality of eggshells, giving 20016 eggs which were considered fertile and suitable for hatching (Table 4).

3.6.1. Fertility

In this study the mean fertility percentage was 88.60 ± 0.55 for RIR eggs. Less fertility percentage (83.53%) was recorded in the 1st hatch group whereas highest fertility percentage (91.08%) was recorded in the 16th hatch group. The findings of Islam *et al.* [50] for fertility percentage (88.29%) support the current results. Ashraf *et al.* [6] in Faisalabad, Pakistan reported higher values of fertility percentage (96.87 ± 1.53). Studies conducted in other countries like Tanzania also recorded higher (91.1%) fertility [51]. In contrast, lower figures for percentage fertility i.e., 53.06% and 78.26% were recorded in Peshawar and Rawalpindi, Pakistan [12, 23]. The higher percentage fertility of RIR can be attributed to its genetic makeup and capability of transferring this trait to the next generations. In the present study, there was a slight variation in fertility levels between the sixteen egg hatch groups. Various studies have shown that fertility within the same breed can vary due to a variety of factors such as management and male-to-female ratio [51].

3.6.2. Hatchability

The mean percentage hatchability of the fertile eggs and all eggs in the egg set for RIR was found out to be 93.48 ± 0.31 and 82.85 ± 0.76%, respectively (Table 4). The results of the current study are in contrast with that of Farooq *et al.* [12] who recorded hatchability percentages of 80.77 ± 0.10 and 42.86 ± 0.07 for fertile eggs and total eggs set, respectively, in Peshawar, Pakistan. Hatchability is determined by fertility and egg quality. Among fertile eggs, the mean hatchability for the RIR breed was revealed to be 64.0% in Tanzania [51], which is lower as compared to the hatchability percentage of RIR reported in the current study. Islam *et al.* [50] also presented a lower hatchability percentage of fertile eggs (88.37%) as compared to all eggs (79.57%). According to the current study, the hatchability of fertile eggs is higher than the hatchability of all eggs. The higher hatchability percentage for RIR recorded in the present study could be attributed to the good management factors of egg collection,

Table 4. Percentage of fertility and hatchability in Kashmiri RIR for year 2022.

Date of hatch	Eggs set (n)	Fertile eggs (n)	Chicks hatched (n)	Fertility (%)	Hatchability of fertile eggs (%)	Hatchability of all eggs (%)
2nd Feb	1500	1253	1145	83.53	91.38	76.33
9th Feb	1500	1286	1175	85.73	91.37	78.33
16th Feb	1500	1317	1225	87.80	93.01	81.67
23rd Feb	1500	1303	1193	86.87	91.56	79.53
2nd Mar	1500	1331	1250	88.73	93.91	83.33
9th Mar	1500	1318	1240	87.87	94.08	82.67
16th Mar	1500	1349	1281	89.93	94.96	85.40
23rd Mar	1500	1363	1292	90.87	94.79	86.13
30th Mar	1500	1357	1283	90.47	94.55	85.53
6th Apr	1500	1332	1236	88.80	92.79	82.40
13th Apr	1200	1035	955	86.25	92.27	79.58
20th Apr	1200	1057	996	88.08	94.23	83.00
27th Apr	1300	1176	1114	90.46	94.73	85.69
5th May	1300	1178	1110	90.61	94.23	85.38
11th May	1300	1177	1106	90.54	93.97	85.08
18th May	1300	1184	1112	91.08	93.92	85.54
Total	22600	20016	18713	88.60	93.48	82.85

handling, and storage on the farm level as the farmers with formal education in poultry husbandry were employed on poultry farm. Hatchability is also strongly influenced by egg size. Eggs that are too big or too little have a low hatchability rate and cause problems during the process of incubation [12]. Temperature and humidity, as well as egg turning, were well managed during the incubation process throughout the experimental period, resulting in better hatchability results in this study.

3.7. Correlation among Different Hatchability Traits

The correlation assessment between various hatchability traits for Kashmiri RIR breed ranged from -0.914 to 0.938. Significant positive correlations were observed between age/fertility (0.712, P = 0.002), age/hatchability of fertile eggs (0.561, P = 0.0235), age/hatchability of all eggs (0.681, P = 0.0037), fertility/hatchability of fertile eggs (0.857, P < 0.0001), fertility/hatchability of all eggs (0.982, P < 0.0001) hatchability of fertile eggs/ hatchability of all eggs (0.938, P < 0.0001) whereas, significant negative correlations were assessed between traits like age/dead in germ (-0.748, P = 0.0009), shape index/dead in shell (-0.798, P = 0.0317), dead in germ/fertility (-0.748, P = 0.0009), dead in germ/hatchability of fertile eggs (-0.505, P < 0.0001) and dead in germ/hatchability of all eggs (-0.540, P < 0.0001), dead in shell/hatchability of fertile eggs(-0.5036, P = 0.0468), dead in shell/ hatchability of all eggs (-0.5401, P = 0.0309) for Kashmiri RIR breed. The rest of all the correlations were found to be non-significant (Table 5).

The present study assessed nonsignificant negative correlations of egg weight/hatchability of fertile eggs (-0.410) and egg weight/hatchability of all eggs set (-0.526) which is in accordance with the findings of Farooq *et al.* [12]. This means that a higher egg weight may result in reduced hatchability. Medium sized eggs ranging from 51 to 55 g have higher fertility as compared to much larger or smaller eggs [52]. Verma *et al.* [44] mentioned positive correlations between shape index and hatchability of all eggs set for Aseel (0.986) and Kadaknath hens (0.998) which is in contrast with the negative correlations of shape index with hatchability of fertile eggs (-0.288) and hatchability of all eggs (-0.526) in Kashmiri RIR reported in this study. The findings of the present study suggest that increased egg length and width (resulting in higher shape index %) would result in decreased hatchability. Similar results were recorded by Farooq *et al.* [12] for RIR breed. The results obtained by Islam *et al.* [50] were in support of the present study as they also observed positive correlations of percentages of fertility with hatchability of fertile eggs and all eggs as well in RIR. Verma *et al.* [44] also reported positive correlations of fertility with hatchability of fertile eggs and hatchability of all eggs in (0.953, 0.980) and (0.968, 0.992) at P<0.01. The positive correlations of fertility and hatchability suggest that increased fertility ensures increased hatchability. This was also observed by Farooq *et al.* [12] in RIR hens.

This study was conducted under controlled conditions at a single government poultry farm, which may not fully reflect variability in rural or

Table 5. Correlation among different hatchability traits in Kashmiri RIR breed.

	Age	Egg weight	Shape index	Fertility	Dead in germ	Dead in shell	Hatchability of fertile eggs	Hatchability of all eggs
Age	1							
Egg weight	-0.659*	1						
Shape index	-0.041	-0.071	1					
Fertility	0.712**	-0.558	-0.2628	1				
Dead in germ	-0.748***	0.444	0.0902	-0.748***	1			
Dead in shell	-0.396	0.035	-0.797*	-0.533*	0.495	1		
Hatchability of fertile eggs	0.561*	-0.410	-0.288	0.857***	-0.914***	-0.504*	1	
Hatchability of all eggs	0.681**	-0.526	-0.284	0.982***	-0.834***	-0.540**	0.938***	1

*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001

commercial settings. Additionally, the sample size for some parameters, such as hatchability correlations, may limit the generalizability of the findings. Future studies should include multiple locations and larger sample sizes to validate these results.

4. CONCLUSIONS

In conclusion, the Kashmiri Rhode Island Red (RIR) breed has demonstrated strong adaptability and potential for both rural and commercial poultry systems in AJK. The breed shows consistent growth, optimal sexual maturity, and high-quality egg production, including favorable traits such as shell thickness, albumen height, and yolk colour. Furthermore, its fertility and hatchability rates confirm its viability under intensive management. The breed's adaptation to local environmental conditions makes it a valuable resource for improving protein production and supporting sustainable poultry farming in the region.

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6. ETHICAL STATEMENT

The ethical committee of the Board of Advanced Studies and Research of the University of Azad Jammu and Kashmir, Muzaffarabad, has approved this study with notification No. F-BASR/(82nd M)/19(i)-48/1609-10/2022, which is in accordance with the rules and regulations.

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Research Article

Preliminary Checklist, Distribution and Host Plants of Family Aphididae (Insecta: Hemiptera) from Azad Jammu and Kashmir, Pakistan

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Abstract: The northern areas of Pakistan, located at the junction of the Oriental and Palaearctic regions, are expected to harbor a rich diversity of flora and fauna. Azad Jammu & Kashmir, situated at the junction of these two regions with multiple climatic zones, extensive forest types, and rich plant diversity, remains underexplored in terms of aphid fauna. This study, based on comprehensive field surveys conducted in the Poonch division from 2015 to 2016, identified 69 aphid species in 39 genera and 10 tribes under seven subfamilies. These aphid species were associated with 71 host plant species under 59 genera and 39 families. The study also provided the first preliminary checklist of aphids in Azad Jammu & Kashmir, documenting their distribution and associated host plants.

Keywords: Aphids, Checklist, Distribution, Host Plants, Oriental, Palaearctic, South Asia.

1. INTRODUCTION

Notwithstanding their notoriety, as the serious pests of agroecosystems [1-3] aphids remain one of the understudied taxa in Pakistan: predominantly an agriculture economy. Pakistan's aphidofauna comprises over 300 species [4] at present constituting about 6% of world's Aphidoidea, mainly reflects the fragmented and inconsistent exploratory surveys, rather than plantlice paucity in the country. The latter comprehensive checklist has been preceded and succeeded by several parochial moderate checklists viz., [5, 6, 8, 10, 11, 26, 27]. The comprehensive work referred to above has been preceded and succeeded by several, moderate checklists [5-15]. These surveys have mainly concentrated on certain regions of the Punjab province and few pockets of Khyber Pakhtunkhawa. Azad Jammu and Kashmir (AJ & K), a biodiversity rich region in the northeast of Pakistan, prior to present study, remained as one of the aphidologically uncharted territories in the country. Present study provides preliminary checklist, distribution and host plants of the studied Aphididae from Poonch division, AJ & K, Pakistan.

2. MATERIAL AND METHODS

Checklist is based on our previous published papers [12-15] and directly collected and identified aphid species from the surveyed area during 2015-2016. In field, we randomly collected the adult specimens during 2015-2016 from cultivated and

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wild flora of different selected localities of Poonch division. Collected specimens were transferred into transparent plastic vials containing 70% ethanol for further studies. Adult female apterae were evaluated taxonomically using the available source of literatures [16-20]. The diagnostic characters of specimens were observed under a Noif Microscope (XSZ 107BN) at 40-100 magnification. Host plants were identified at National Herbarium, National Agriculture Research Center (NARC), Islamabad, Pakistan. The identified species have been deposited in the Laboratory of Biosystematic, Department of Entomology, University of Poonch Rawalakot, Azad Jammu & Kashmir, Pakistan. This paper also presents an annotated preliminary checklist, distributional information, and the host plant of studied Aphididae. The general map of Pakistan showing the territories of State of Azad Kashmir is presented (Figure 1). The map was prepared with ArcGIS 10.5 software (Esri, Redlands, CA) by using the original base map of Pakistan.

3. RESULTS

Taxonomic analysis of the Aphididae material collected during 2015-2016 from Poonch division resulted in 69 species in 39 genera stretched over seven subfamilies and 10 tribes. These species were found associated with a total of 71 species, 59 genera and 39 families of host plants**.** Taxonomy, distribution and host plants of the studied Aphididae are presented below:

Family APHIDIDAE Latreille, 1802
Subfamily APHIDINAE Latreille, 1802
Tribe APHIDINI Latreille, 1802
Subtribe APHIDINA Latreille, 1802
Genus *Aphis* Linnaeus, 1758

1. *Aphis affinis* del Guerico, 1911
Previous records in Azad Jammu and Kashmir: District Poonch (Abbaspur, Alisojal, Hajira, Haveli, Rawalakot) [12, 15].
Host plants: *Mentha spicata*, *Mentha longifolia,* Mentha *requienii* [4, 12, 15].

2. *Aphis craccivora* Koch, 1854
Previous records in Azad Jammu and Kashmir: District Bagh (Singola), District Poonch (Abbaspur, Alisojal, Banjosa, Hajira, Haveli, Hussainkot, Khaigala, Paniola, Rawalakot, Thorar, Tolipir), District Sudhanoti (Mang, Palandari, Thrar Khal) [12-15].
Host plants: *Alhagim aurorum*, *Amaranthus viridis*, *Coriandrum sativum*, *Bidens biternate*, *Dalbergia sissoo*, *Dendranthema indicum*, *Cassia fistula*, *Cajanus indicus*, *Cassia surratensis*, *Parthenium*

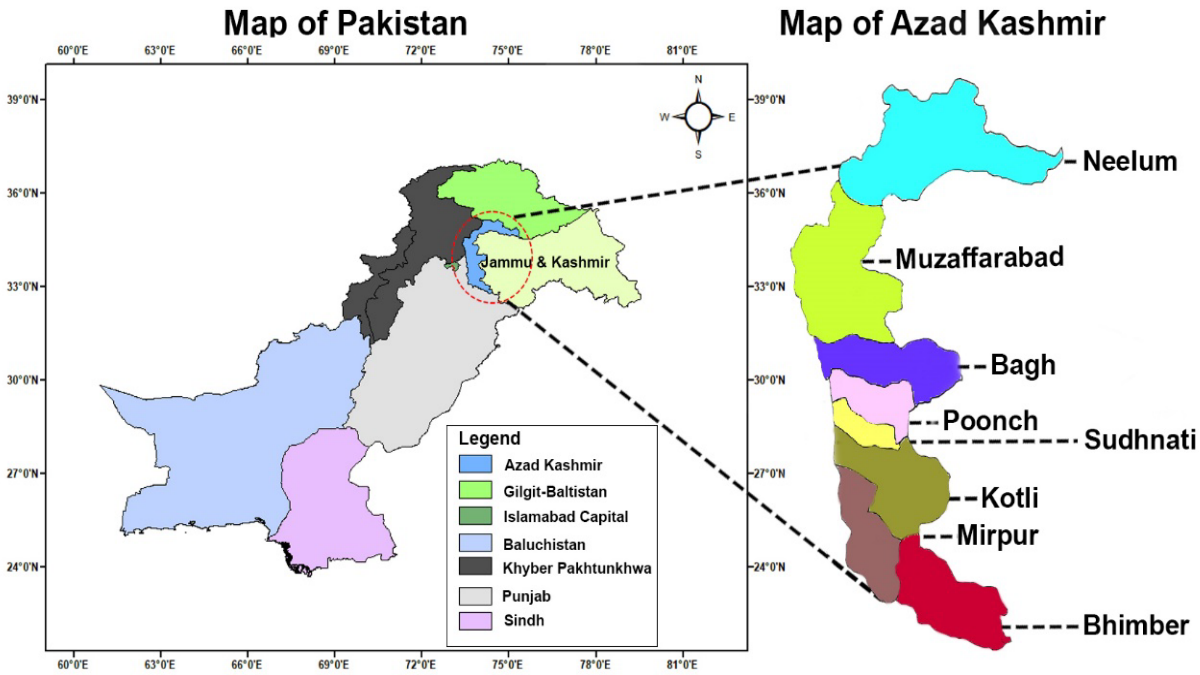

Fig. 1. General map of Pakistan showing the territories of State of Azad Kashmir.

hysterophorus, *Melilotus indica*, *Robiniapseudo acacia*, *Sesbania aegyptiaca*, *Tephrosia purpurea*, *Trigonella foenum-graecum*, *Vicia faba* , *Alcea rosea*, *Hibiscus* sp., *Ficus carica*, *Psidium guajava*, *Bougainvillea* sp., *Punica granatum*, *Eriobotrya japonica*, *Pyrus malus*, *Salix* sp., *Ailanthus altissima*, *Cestrum* sp., *Solanum nigrum*, *Tribulus terrestris* [4, 12-14].

3. *Aphis fabae* Scopoli, 1763
Previous records in Azad Jammu and Kashmir: District Bagh (Singola), District Poonch (Abbaspur, Alisojal, Banjosa, Datoot, Hajira, Haveli, Hussainkot, Khaigala, Paniola, Rawalakot, Thorar, Tolipir), District Sudhanoti (Mang, Palandari, Thrar Khal) [12, 14, 15].
Host plants. *Alcea rosea*, *Amaranthus viridis*, *Coriandrum sativum*, *Daucus carota*, *Conyza canadensis*, *Galinsoga perviflora*, *Parthenium hysterophorus*, *Sonchus asper*, *Tagetes minuta*, *Cyperus rotundus*, *Euphorbia* sp., *Leucaena leucocephala*, *Taxaracum officinale*, *Lonicera quinquelocularis*, *Rumex acetosa*, *R. dentatus*, *Cestrum nocturnum*, *Solanum nigrum*, *Withania somnifera*, *Peganum harmala* [4, 12, 14].

4. *Aphis gossypii* Glover, 1877
Previous records in Azad Jammu and Kashmir: District Bagh (Singola), District Poonch (Abbaspur, Alisojal, Banjosa, Datoot, Khaigala, Hajira, Haveli, Hussainkot, Rawalakot, Thorar), District Sudhanoti (Mang, Palandari, Trar Khel) [12, 14, 15].
Host plants: *Althea rosea, Amaranthus viridis, A. altisima, Chenopodum album, Conyza canadensis, Coriandrum sativum, Datura alba, Dendrathema indicum, Galinosoga parviflora, Hibiscus rosa-sinensis, Leucas cephalotes, Papaver somniferrum, Parthenium hysterophorus, Plantago major, Solanum nigrum, Sonchus asper, Tagetes minuta, Taraxacum officinale, Vicia sativa* [12-14].

5. *Aphis nasturtii* Kaltenbach, 1843
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Hajira, Haveli, Hussainkot, Rawalakot) [12, 15].
Host plants: *Chenopodum album, Dendranthema indicum, Viola biflora, Tropaeolum majus* [12].

6. *Aphis nerii* Boyer de Fonscolombe, 1841
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Abbaspur, Alisoja, Hajira, Rawalakot, Thorar), District Sudhanoti (Mang, Palandari) [14, 15].
Host plant: *Malus pumila* [14].

7. *Aphis pomi* de Geer, 1773
Previous records in Azad Jammu and Kashmir: District Bagh (Singola), District Poonch (Abbaspur, Alisoja, Banjosa, Datoot, Hajira, Hussainkot, Khaigala, Paniola, Rawalakot, Taolipir), District Sudhanoti (Mang, Pallandri) [14, 15].
Host plant: *Malus pumila* [4, 14].

8. *Aphis punicae* Passerini, 1863
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Abbaspur, Banjosa, Hajira, Rawalakot, Thorar), District Sudhanoti (Mang, Pallandri) [14, 15].
Host plant: *Punica granatum* [14].

9. *Aphis spiraecola* Patch, 1914
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Abbaspur, Banjosa, Haveli, Hajira, Khaigala, Rawalakot, Thorar), District Sudhanoti (Mang, Pallandri, Thrar Khal) [12, 14, 15].
Host plant: *Eriobotrya japonica* [14].

10. *Aphis* (*Toxoptera*) *aurantii* Boyer de Fonscolombe, 1841
Previous records in Azad Jammu and Kashmir: District Poonch (Abbaspur, Hajira, Thorar) [14, 15].
Host plant: *Citrus* sp. [14].

11. *Aphis* (*Toxoptera*) *citricidus* (Kirkaldy, 1907)
Previous records in Azad Jammu and Kashmir: District Poonch (Abbaspur, Hajira, Thorar) [15].
Host plant: *Citrus sinensis* [15].

Subtribe RHOPALOSIPHINA Mordvilko, 1924
Genus *Hysteroneura* Koch, 1854

12. *Hysteroneura setariae*(Thomas, 1878)
Previous records in Azad Jammu and Kashmir: District Poonch (Abbaspur, Haveli, Hajira) [12].
Host plant: *Cynodon dactylon* [12].

Genus *Rhopalosiphum* Koch, 1854

13. *Rhopalosiphum maidis* (Fitch, 1856)
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh, Singola), District Poonch (Abbaspur, Ali Sojal, Banjosa, Datoot, Hajira,

Hussainkot, Khaigala, Paniola, Rawalakot, Thorar), District Sudhnoti (Mang, Palandari, Trar Khel) [15].
Host plants: *Sorghum bicolor*, *Zea mays*

14. *Rhopalosiohum nymphaeae* (Linnaeus, 1761)
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Alisojal, Banjosa, Hajira, Hussainkot, Khaigala, Rawalakot) [14, 15].
Host plants: *Prunus domestica, P. armeniaca, Typha* sp. (water lily) [14].

15. *Rhopalosiphum padi* (Linnaeus, 1758)
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh, Singola), District Poonch (Abbaspur, Ali Sojal, Banjosa, Datoot, Hajira, Hussainkot, Khaigala, Paniola, Rawalakot, Thorar), District Sudhnoti (Mang, Palandari, Trar Khel) [15].
Host plant: *Rhopalosiphum padi* was found occurring as pest of corn in all the study area.

Genus *Schizaphis* Börner, 1931

16. *Schizaphis graminum* (Rondani, 1852)
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh, Singola), District Poonch (Abbaspur, Banjosa, Datoot, Hajira, Hussainkot, Khaigala, Paniola, Rawalakot, Thorar), District Sudhnoti (Mang, Palandari, Trar Khel) [15].
Host plants. *Schizaphis graminum* mostly found on *Cynodon dactylon*, *Hordeum vulgare*, *Triticum aestivum*.

Tribe MACROSIPHINI Wilson, 1910
Subtribe MACROSIPHINA Wilson, 1910
Genus *Acyrthosiphon* Mordvilko, 1914

17. *Acyrthosiphon gossypii* Mordvilko, 1914
Previous records in Azad Jammu and Kashmir: District Bagh (Singola), District Poonch (Abbaspur, Alisojal, Banjosa, Datoot, Khaigala, Hajira, Haveli, Hussainkot, Rawalakot, Thorar), District Sudhanoti (Mang, Palandari, Trar Khel) [12-15].
Host plants. *Phaseolus vulgaris*, *Trigonella* sp. and *Vicia* sp. were recorded from study area.

18. *Acyrthosiphon ghanii* Eastop, 1971
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh city, Singola), District Poonch (Datoot, Rawalakot), District Sudhnoti (Mang, Palandari, Trar Khel) [15].
Host plants. *Scorzonera subaphylla*, *Sonchus oleraceus* [4, 15].

19. *Acyrthosiphon malvae* (Mosley, 1841)
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh, Singola), District Poonch (Abbaspur, Alisojal, Banjosa, Datoot, Hajira, Haveli, Hussainkot, Khaigala, Paniola, Rawalakot, Thorar, Tolipir), District Sudhnoti (Palandari) [12, 15].
Host plants: *Althea rosea*, *Tropaeolum majus* [12].

20. *Acyrthosiphon pisum* (Harris, 1776)
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh, Singola), District Poonch (Abbaspur, Alisojal, Banjosa, Datoot, Hajira, Haveli, Hussainkot, Khaigala, Paniola, Rawalakot, Thorar, Tolipir), District Sudhnoti (Mang, Trar Khel) [12, 15].
Host plants: *Lathyrus odaratus*, *Malva perviflora* [12].

Genus *Aulocorthum* Mordvilko, 1914

21. *Aulacorthum solani* (Kaltenbach, 1843)
Previous records in Azad Jammu and Kashmir: District Bagh (Singola), District Poonch (Hajira, Haveli, Hussainkot, Khaigala, Rawalakot, Thorar) [12].
Host plants: *Chenopodum album*, *Conyza canadensis*, *Galinosoga perviflora*, *Plantago major*, *Silybum marianum*, *Sonchus oleraceous*, *Ricinus communis*, *Coriandrum sativum*, *Tagetes minuta* [12].

Genus *Berberidaphis* Narzikulov, 1960

22. *Berberidaphis lydiae* Narzikulov,1957
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [14, 15].
Host plants: *Berberis lyceum* [14]. *Berberidaphis lydiae*, a central Asian species, first reported from Northern areas of Pakistan at elevations ranging from 1700-2600 m ASL [4].

Genus *Brachycaudus* Van der Goot, 1913

23. *Brachycaudus cardui* (Linnaeus, 1758)
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [15].
Host plants. *Denndrathema indicum, Cirsium* sp., *Senecio* sp., *Cnicus wallichii* [4, 15, 21].

24. *Brachycaudus helichrysi* (Kaltenbach, 1843)
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Alisoja, Banjosa, Hajira, Haveli, Hussainkot, Khaigala, Rawalakot, Thorar), District Sudhnoti (Mang) [12, 14, 15].
Host plants: *Galinsoga perviflora*, *Plantago major*, *Prunus domestica*, *Prunus armeniaca*, *Silybum marianum*, *Sonchus oleraceus*, *Tagetes minuta*, *Vicia sativa*, *Viola biflora* [12, 14].

Genus *Brevicoryne* Das, 1915

25. *Brevicoryne brassicae* (Linnaeus, 1758)
Previous records in Azad Jammu and Kashmir: District Bagh (Singola), District Poonch (Abbaspur, Banjosa, Datoot, Hajira, Khaigala, Paniola, Tolipir), District Palandri, District Sudhnoti (Mang, Trar Khel) [15,).
Host plant: *Brassica compestris.*

Genus *Capitophorus* van der Goot, 1913

26. *Capitophorus carduinus* (Walker, 1850)
Previous records in Azad Jammu and Kashmir: District Poonch (Khaigala, Rawalakot) [12, 15].
Host plant: *Silybum marianum* [12].

Genus *Chaetosiphon* Mordvilko, 1914

27. *Chaetosiphon* (*Pentatrichopus*) *fragaefolii* (Cockerell, 1901)
Previous records in Azad Jammu and Kashmir: District Poonch (Alisojal, Banjosa, Khaigala, Rawalakot) [13, 15].
Host plant: *Rosa* sp. [13].

28. *Chaetosiphon* (*Pentatrichopus*) *tetrarhodum* (Walker, 1849)
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Alisojal, Banjosa, Khaigala, Rawalakot) [13, 15].
Host plant: *Rosa* sp.

29. *Chaetosiphon* (*Pentatrichopus*) *thomasi* Hille Ris Lambers, 1953
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Khaigala, Rawalakot) [13, 15].
Host plant: *Rosa* sp. [13].

Genus *Dysaphi*s Börner, 1931

30. *Dysaphistulipae*(BoyerdeFonscolombe,1841)
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [4, 15].
Host plants. *Iris* sp. [4]. During present study this species recorded on *Gladiolus grandiflora* Linnaeus from Rawalakot and Bagh.

Genus *Hyadaphis* Kirkaldy, 1904

31. *Hyadaphis coriandri* (Das, 1918)
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [15].
Host plants: *Coriandrum sativum* [12], *Crocus sativus* (present study).

Genus *Hyperomyzus* Börner, 1933

32. *Hyperomyzus carduellinus* (Theobald, 1915)
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh), District Poonch (Banjosa, Hajira, Haveli, Rawalakot, Thorar, Tolipir), District Sudhanoti (TrarKhel) [12, 15].
Host plant: *Sonchus oleraceous* [12] present study.

33. *Hyperomyzus lactucae* (Linnaeus, 1758)
Previous records in Azad Jammu and Kashmir: District Poonch (Datoot, Hajira, Khaigala, Rawalakot), District Sudhanoti (Trar Khel) [15].
Host plants. *Sonchus* sp. [4]. During present study this species reported from *Sonchus oleraceous*.

34. *Hyperomyzus pallidus* Hille Ris Lambers, 1935
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh), District Poonch (Datoot, Khaigala, Hajira, Rawalakot), District Sudhanoti (Trar Khel) [15].
Host plants. *Ribes* sp. (Grossulariaceae), *Sonchus oleraceus* [15].

Genus *Liaphis* Mordvilko, 1928

35. *Liaphis erysimi* (Kaltenbach, 1830)
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [15].
Host plants. *Brassica napus*, *B. oleracea*, *B. nigra*, *B. rapa*, *B. oleracea* and *B. acephala* in recorded as host plant during the present study.

Genus *Macrosiphoniella* Del Guercio, 1911

36. *Macrosiphoniella sanborni* (Gillete, 1908)
Previous records in Azad Jammu and Kashmir:

District Poonch (Ali Sojal, Rawalakot) [15].
Host plant: *Chrysanthemum* sp. [11, 21].

Genus *Macrosiphum* Passerini, 1860

37. *Macrosiphum euphorbiae* (Thomas, 1878)
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh, Singola), District Poonch (Abbaspur, Alisojal, Banjosa, Datoot, Khaigala, Hajira, Haveli, Hussainkot, Paniola, Rawalakot, Thorar, Tolipir), District Sudhanoti (Mang) [12, 13, 15].
Host plants: *Althea rosea*, *Galinosoga perviflora*, *Ricinus communis*, *Rosa indica*, *R. chinensis*, *Rumex acetosa*, *Sonchus asper*, *S. oleraceus*, *Tagetes minuta*, *Viola biflora* [12, 13].

38. *Macrosiphum gei* (Koch, 1855)
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [15].
Host plant: *Coriandrum sativum*.

39. *Macrosiphum rosae* Linnaeus, 1757
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh, Singola), District Poonch (Abbaspur, Alisojal, Banjosa, Datoot, Hajira, Haveli, Hussainkot, Khaigala, Rawalakot, Thorar, Tolipir), District Sudhnoti (Mang, Palandari, Trar Khel) [12, 13, 15].
Host plants: *Rosa chinensis*, *R. indica* [12, 13].

Genus *Metopolophium* Mordvilko, 1914

40. *Metapolophium dirhodum* (Walker, 1849)
Previous records in Azad Jammu and Kashmir: District Bagh (Bagh, Singola), District Poonch (Abbaspur, Alisojal, Banjosa, Khaigala, Paniola, Rawalakot, Tolipir), District Sudhanoti (Mang, Trar Khel) [13, 15].
Host plant: *Rosa* sp. [13].
Remarks: *Metopolophium dirhodum* was already reported for Pakistan by [4]. Hassan *et al*. [9] reported this species on secondary host, *Triticum* sp. from northern areas of Pakistan. Present study gives secondary host *Rosa chinensis*.

41. *Metapolophium montanum* Hille Ris Lambers, 1966
Previous records in Azad Jammu and Kashmir: District Poonch (Alisojal, Banjosa, Rawalakot, Khaigala, Paniola, Tolipir), District Sudhanoti (Mang) [13, 15].
Host plant: *Rosa* sp. [13].

Genus *Myzaphis* Van der Goot, 1913

42. *Myzaphis bucktoni* (Jacob 1946)
Previous records in Azad Jammu and Kashmir: District Poonch (Alisojal, Khaigala, Rawalakot) [13, 15].
Host plant: *Rosa moschata*. [13].

43. *Myzaphis rosarum* (Kaltenbach, 1843)
Previous records in Azad Jammu and Kashmir: District Poonch (Abbaspur, Ali Sojal, Banjosa, Datoot, Hussainkot, Khaigala, Rawalakot, Thorar, Tolipir), Sudhanoti (Palandari) [13, 15].
Host plant: *Rosa* sp. [13].

44. *Myzaphis turanica* (Nevsky 1929)
Previous records in Azad Jammu and Kashmir: District Poonch (Khaigala, Rawalakot) [13, 15].
Host plant: *Rosa* sp. [13].

Genus *Myzus* Passerini, 1860

45. *Myzus ascalonicus* (Doncaster 1946)
Previous records in Azad Jammu and Kashmir: District Poonch (Khaigala, Rawalakot) [15].
Host plants: *Brassica pekinensis*, *Solanum tuberosum* [4]. New host plant, *Bidens biternata* and *Brassica oleraceava* recorded from Azad Jammu and Kashmir.

46. *Myzus ornatus* (Laing 1932)
Previous records in Azad Jammu and Kashmir: District Bagh, Poonch (Abbaspur, Hajira, Rawalakot, Thorar), District Sudhanoti (Mang, Trar Khel) [12, 14, 15].
Host plants: *Bidens biternata, Sonchus asper, Tecoma stans* [12, 14].

47. *Myzus persicae* (Sulzer, 1776)
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Abbaspur, Alisojal, Banjosa, Datoot, Hajira, Hussainkot, Paniola, Thorar, Rawalakot), District Sudhanoti (Palandari, Mang, Trar Khel) [12, 14, 15].
Host plants: *Althea rosea*, *Amaranthus viridis*, *Chenopodum album*, *Conyza canadensis*, *Galinosoga perviflora*, *Tagetes minuta*, *Dendranthema indicum*, *Bidens bitternata*, *Plantago major*, *Prunus persica*, *Ricinus communis*, *Rumex acetosa*, *Solanum nigrum*, *Sonchus asper*, *S.*

oleraceous, *Viola bioflora* [12, 14].

Genus *Nasonovia* Mordvilko, 1914

48. *Nasonovia ribisnigri* (Mosley, 1841)
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Banjosa, Rawalakot) [15].
Host plant: *Sonchus oleraceus* [15].

Genus *Neomyzus* Van der Goot, 1915

49. *Neomyzus circumflexus* (Buckton, 1876)
Previous records in Azad Jammu and Kashmir: District Poonch (Abbaspur, Ali Sojal, Rawalakot) [15].
Host plant: *Sonchus oleraceous*.

Genus *Phorodon* Passerini, 1860

50. *Phorodon cannabis* Passerini, 1860
Previous records in Azad Jammu and Kashmir: District Poonch (Ali Sojal, Hajira, Haveli) [12].
Host plant: *Cannabis sativa* [12].

Genus *Pterocomma* Buckton, 1879

51. *Pterocomma beulahense* (Cockerell, 1904)
Previous records in Azad Jammu and Kashmir: District Poonch (Hajira) [14, 15].
Host plant: *Populus ciliata* [14].

Genus *Rhodobium* Hille Ris Lambers, 1947

52. *Rhodobium porosum* (Sanderson, 1900)
Previous records in Azad Jammu and Kashmir: District Bagh, District Poonch (Hajira, Haveli), District Sudhnoti (Mang) [13, 15].
Host plant: *Rosa* sp. [13].

Genus *Semiaphis* Van Der Goot, 1913

53. *Semiaphis heraclei* (Takahashi, 1921)
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [15].
Host plant: *Coriandrum sativum* [4, 15].

Genus *Sitobion* Mordvilko, 1914

54. *Sitobion africanum* (Hille Ris Lambers, 1954)
Previous records in Azad Jammu and Kashmir: District Poonch (Hajira, Thorar) [15].
Host plant: *Sitobion africanum* was collected on wild *Ficus sp*. at Hajira [15].

Genus *Uroleucon* Mordvilko, 1914

55. *Uroleucon ambrosiae* (Thomas, 1878)
Previous records in Azad Jammu and Kashmir: District Poonch (Hajira, Thorar, Rawalakot) [15].
Host plants: *Conyza canadensis*, *Sonchus asper* [15].

56. *Uroleucon compositae* (Theobald, 1915)
Previous records in Azad Jammu and Kashmir: District Bagh (Singola), District Poonch (Ali Sojal, Hajira, Khaigala, Rawalakot, Thorar), District Sudhnoti (Mang) [12, 15].
Host plants: *Cercis canadensi*, *Sonchus asper*, *Tagetes minuta* [12].

Genus *Wahlgreniella* Hille Ris Lambers, 1949

57. *Wahlgreniella nervata* (Gillete, 1966)
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [12, 13, 15].
Host plant: *Rosa* sp. [13].

Subfamily CALAPHIDINAE Oestlund, 1919
Tribe PANAPHIDINI Oestlund, 1923
Genus *Chromaphis* Walker, 1870

58. *Chromaphis juglandicola* (Kaltenbach, 1843)
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [14, 15].
Host plant: *Juglans regia* [14].

Genus *Panaphis* Kirkaldy, 1904

59. *Panaphis juglandi* (Goeze, 1778)
Previous records in Azad Jammu and Kashmir: District Poonch (Rawalakot) [14].
Host plant: *Juglans regia* [14].

Subfamily CHAITOPHORINAE Mordvilko, 1908
Tribe CHAITOPHORINI Mordvilko, 1908
Genus *Chaitophorus* Koch, 1854

60. *Chaitophorus pakistanicus* Hille Ris Lambers, 1966
Previous records in Azad Jammu and Kashmir: District Poonch (Hajira, Thorar) [14].

Host plant: *Salix acmophylla* [14].

Tribe SIPHINI Mordvilko, 1928
Genus *Sipha* Passerini, 1860

61. *Sipha* (*Rungsia*) *maydis* Passerini, 1860
Previous records in Azad Jammu and Kashmir: District Poonch (Hajira) [15].
Host plants. *Cynodondactylon*, *Hordeum murinum*, *Hordeum vulgare*, *Triticum aestivum*, *Sorghum bicolor*, *Phalaris arundinacea*, *Polypogon fugax*, *Zea mays* [22, 23, 24]. We found dense colonies of this species on upper surface of the leaf-blades of *Arundo donax* in Hajira. Lower surface of leaves were covered with exuded sap and ant attendance was also noted.

Subfamily ERIOSOMATINAE Kirkaldy, 1905
Tribe FORDINI
Subtribe MALAPHIDINA
Genus *Melaphis* Walsh, 1867

62. *Melaphis rhois* (Fitch, 1866)
Previous record in Azad Jammu and Kashmir: Poonch (Hajira).
Host plant: *Rhus javanica*.

Subfamily GREENIDEINAE Baker, 1920
Tribe GREENIDEINI Baker, 1920
Genus *Greenidea* Schouteden, 1905

63. *Greenidea* (*Trichosiphum*) *psidii*van der Goot, 1917
Previous records in Azad Jammu and Kashmir: District Poonch (Abbaspur) [14, 15].
Host plant: *Psidium guajava* [14].

64. *Greenidea* (*Greenidea*) *ficicola* Takahashi, 1921
Previous record in Azad Jammu and Kashmir: District Poonch (Hajira) [14, 15].
Host plant: *Ficus religiosa* [14].

Subfamily HORMAPHIDINAE Mordvilko, 1908
Tribe CERATAPHIDINI Baker, 1920
Genus *Astegopteryx* Karsch, 1890

65. *Astegopteryx bambusae* (Buckton, 1893)
Previous record in Azad Jammu and Kashmir: District Poonch (Abbaspur) [14].
Host plant: *Bambusa* sp. [14].

Subfamily LACHNINAE Herrich-Schaeffer, 1854
Tribe EULACHNINI Baker, 1920
Genus *Cinara* Curtis, 1835

66. *Cinara tujafilina* (del Guericio, 1909)
Previous record in Azad Jammu and Kashmir: District Poonch (Hajira, Rawalakot) [15].
Remarks:*Cinara tujafilina* was first reported on *Thuja* sp. for Pakistan [4] on foot hills from 300-2000 m ASL. We found this species on *T. orientalis* and *T. chinensis* in Rawalakot and Bagh.
Host plant: Recorded this species on *T. orientalis* and *T. chinensis* in Rawalakot and Bagh.

67. *Cinara maculipes* Hille Ris Lambers, 1966
Previous record in Azad Jammu and Kashmir: District Poonch (Rawalakot) [14, 15].
Host plant: *Pinus wallichiana* [14].

Tribe LACHNINI Herrich-Schaeffer, 1854
Subtribe LACHNINA Herrich-Schaeffer, 1854
Genus *Lachnus* Burmeister, 1835

68. *Lachnus tatakaensis* Takahashi, 1937
Previous records in Azad Jammu and Kashmir: District Poonch (Hajira) [15].
Host plant: *Salix sp*.

Genus *Pterochloroides* Mordvilko, 1914

69. *Pterochloroides persicae* (Cholodkovsky 1899)
Previous record in Azad Jammu and Kashmir: District Bagh, District Poonch (Alisojal, Rawalakot), District Sudhnoti (Trar Khel) [14, 15].
Host plant: *Prunus armeniaca* [14].

4. DISCUSSION

The study conducted by Naumann-Etienne and Remaudière [4] marked a pivotal moment in documenting Pakistan's aphididae. Subsequent checklists, such as those by Nasir and Yousaf [8] and Irshad [25], delved into regional surveys, offering insights into the diversity of aphid species across provinces like Punjab. Shahjehan and Tullu [27] focused on Abbottabad, while Bodlah *et al.* [11] explored the rain-fed region of Punjab. Zia *et al*. [10] and Maryam *et al.* [26] targeted the Pothwar and Gujranwala regions, respectively. Maryam *et al.* [26] describe the comprehensive checklist and emphasized the importance of exploring overlooked

regions, shedding light on the potentially rich aphid fauna in Pakistan. The current study's findings are noteworthy, encompassing 69 aphid species across 39 genera spanning seven subfamilies, 10 tribes, and establishing associations with 71 host plant species from 37 genera and 39 families. The emphasis on these areas is crucial for unveiling the hidden diversity of aphids, highlighting the richness that may have been overlooked in prior research efforts.

5. CONCLUSIONS

This paper presents preliminary checklist, distribution and host plants of Aphididae from the Poonch division, Azad Jammu and Kashmir based on published records in recent years data. The study emphasizes the importance of investigating vastly overlooked regions in the country. This checklist would serve as a baseline for the future workers engaged in aphidological persuits not only in the study area but also elsewhere in Pakistan, particularly regions of Punjab and Khyber Pakhtunkhawa provinces geographically adjacent to the study area. On this account the study also suggests updating of related checklists in these latter regions. Due to the study's limited scope, absence of link roads, frequent land sliding, and lack of funding, it is highly likely that numerous exotic and endemic species remained unexplored in this particular research. More extensive works are needed to fully realize the regional aphid diversity of Azad Jammu and Kashmir.

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7. CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest related to this article's publication.

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Research Article

Spatial Assessment of Drinking Water Quality in Mardan City, Khyber Pakhtunkhwa, Pakistan

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Abstract: The quality water is needed for human health. However, most of the water supply agencies focus on only water supply aspect instead of water quality. Majority of the consumers are also not aware of the importance of water quality. In the present study, samples of drinking water were collected from households located in Mardan city. These water samples were evaluated for physical parameters (electrical conductivity (EC), pH, total hardness, total dissolved solids (TDS), and turbidity) and chemical parameters (sulphates, nitrates, sodium, chlorides and potassium ions). The physical parameters (pH, alkalinity, total hardness and TDS) and chemical parameters (sodium, chloride and nitrate) in all the water samples were found according to the guidelines prescribed by WHO for the quality of drinking water. However, this study has shown abnormal conditions regarding physical parameters (turbidity 0.82 NTU and EC 1480 µS/cm) and chemical parameters (potassium 13.7 mg/l and sulphates 512 mg/l) at selected sample areas. These water samples values are higher than the guidelines values recommended by WHO for the quality of drinking water. Therefore, it can be concluded that the groundwater quality at the majority places are safe drinking purposes in the study area.

Keywords: Water Samples, Stratified Sampling, Physio-chemical Analysis, Spatial Distribution, Water Quality.

1. INTRODUCTION

Water is essential for human needs and life processes on the globe [1]. The large part of living things is consist of water [2]. In human beings, three to fourth of its weight consists of water [3]. It is not possible to sustain life on earth without water [4]. The earth approximately consist of 1.4 trillion cubic meter of water [5]. Less than 1 percent of this water is available in the form of rivers and groundwater to meet our requirements [6]. According to a study conducted by United Nations, revealed that population is increasing and the availability of freshwater is declining [7]. It would results in severe shortage of water in South Asia, Africa, and Middle East in the next two decades [8]. Due to rapid population growth and urbanization, not only quantity of water is diminishing but its quality is also deteriorating particularly in the developing countries [9]. Water should not contain any substance which is injurious to human health [10]. Disease-causing microbes, chemical molecules and minerals are the examples of such substances [11]. Many people in developing countries experiencing health problems either due to lack of access to quality water and thus using contaminated water for drinking purposes [12]. About 5 million children mortality occurs each year due to the drinking of contaminated water in the developing countries[13]. Rapid population growth and inadequate water quality management further aggravating this issue [14]. Quality of water supply is considered a serious issue in the developing countries of the world [15]. The people of these

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countries are also unaware of quality drinking water and their impacts on health [16, 17]. These health issues are directly or indirectly related to high or low concentration of the contaminants in the drinking water [18]. Majority of the residents of developing countries are prone to water borne diseases due inadequate and unsafe public water supply [19]. Water borne diseases associated with inadequate and unsafe water supply is responsible approximately for 60% of the health issues in developing countries [20]. The contamination in drinking water changes its chemical properties which is disturbing the overall equilibrium of the system and is resulting its consumption impractical [21]. The improved water supply to the public depends on the analytical assessment of the drinking water. Therefore, analysis of water quality parameters is necessary for the evaluation of water pollution.

The major cause of waterborne diseases is the consumption of contaminated water [22]. The use of contaminated water results in morbidity and mortalities in underdeveloped and developing countries [23]. The infant mortality rate is particularly high in poorer countries [24]. The scientific studies carried out in different parts of Pakistan have reported the contamination of drinking water and its related health problems [25]. Almost 30% of the diseases and 40% of the deaths in Pakistan are caused by poor water quality [26]. Every fifth inhabitant in the country reports having a disease or illness brought on by contaminated water [27]. Diarrhea being the most common cause of mortality in newborns and young children in the country [28]. According to a report, ¼ infant mortality is due to waterborne diseases in dveloping countries [29]. Inspite of this crictical situation, the water supply agencies give less attention to water quality and their emphasis, in most cases, is only on the supply of water [30].

Water quality is an important aspect of drinking supply system. It has a direct effect on the health of its consumers. It not only affects the health of human beings but also aid the economic burden on its consumers. Therefore, the main aim of the current study is to evaluate the drinking water quality of different water samples collected from different union councils of Mardan city, Khyber Pakhtunkhwa, Pakistan and to compare it with WHO standards for drinking water [31].

This study analyze the current quality of drinking water supply to check either it is safe for drinking purpose or not. The results of analysis of drinking water samples will be helpful for the awareness for the people of the study area. Therefore this study evaluate the physio-chemical parameters of drinking water in Mardan City like pH, alkalinity, total dissolved solids, total hardness, electrical conductivity, turbidity, sodium, potassium, chlorides, nitrate and sulphates ions. The whole Mardan city is selected for the purpose of water quality assessment. Sixteen drinking water samples were collected from private bores within the houses located in different neighborhood Councils of Mardan city. These samples were analyzed for various physio-chemical parameters. The spatial distribution of these samples is shown in Figure 1.

2. MATERIALS AND METHODS

2.1. Drinking Water Quality Parameters Tested

Sixteen drinking water quality samples were collected from the study area. These samples were tested for six physical and five chemical parameters. Physical parameters tested were pH, conductivity, total hardness, total dissolved solids (TDS) and total alkalinity.

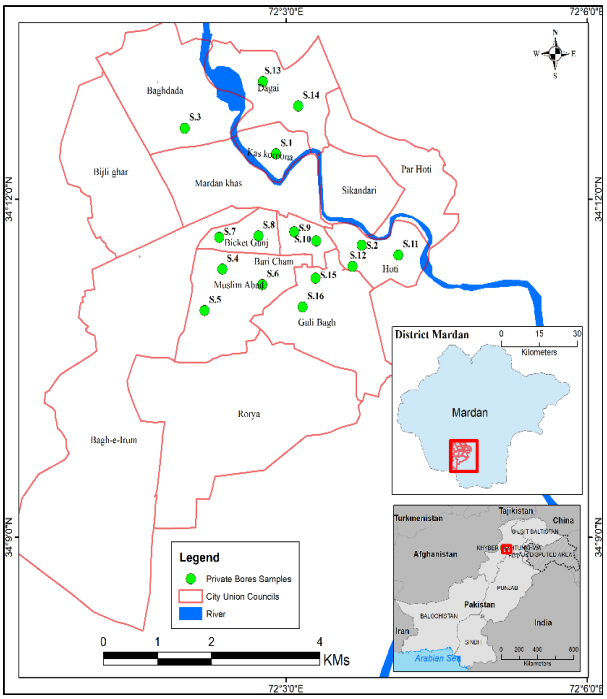

Fig. 1. The map shows the location of the study area and distribution of the locations of water samples.

The solubility and biological availability of chemical elements are determined by the pH of water. Both too high and low concentrations of pH can affect the quality of drinking water. High concentration of pH in drinking water produces bitter taste and deposits in water using equipment's and water pipes. WHO recommends the range of pH from 6.5 to 8.5 for drinking water [31].

Electrical Conductivity (EC) in drinking water is the measures of the capacity of water to conduct electricity. It indicates the presence of impurities in the drinking water in the form minerals, chemicals and dissolved substances. Its concentration is affected by temperature and salinity in the form of dissolved salts. High concentration of EC can affect its odor, taste and quality of drinking water. WHO recommends 400 μS/cm levels of EC for drinking water [31].

Total dissolved solids (TDS) are the presence of inorganic and some organic minerals or salts such as chlorides, potassium, sodium, bicarbonates, magnesium and sulfates etc. These minerals can cause change in taste and in color of water. The high values of TDS indicate that the water is highly mineralized and vice versa. High concentration of TDS may affect persons who are suffering from and heart and kidney diseases. WHO recommends 500 mg/l as a desirable limit and maximum limit of 1000 mg/l of TDS for drinking purpose [31].

The normal range of alkalinity is considered 5 to 125 mg/l for drinking purposes. However, WHO recommends maximum permissible limit of 200 mg/l of alkalinity in drinking water [31]. Total Suspended Solids or Turbidity is caused by sewage matter. The turbidity in water increases the risk pathogenic organisms as they are protected by turbidity particles and escaping them from the disinfectant effect. WHO recommends 5mg/l as a permissible limit in drinking water.

Niterate is one of the most significant diseases causing parameters of water quality. It is responsible for blue baby syndrome in infants. The main sources of nitrate in drinking water are erosion of natural deposits, nitrogen cycle, nitrogenous fertilizers and industrial waste. The WHO allows maximum permissible limit of 50 mg/l of nitrate in drinking water.

The dissolution of sulphuric acids is the main source of sulphates in water and is found in all sources of water. Other important sources of sulphates are mine drainage and oxidation of pyrite in water sources. There are no serious health effects of sulphates in water however, high concentration of sulphates can cause gastrointestinal diseases in human beings. WHO recommends 250 mg/l of sulphates for drinking water. The concentration of sulphates higher than 500 mg/l should be reported to the health authorities.

The dissolution of salts of hydrochloric acid is the main source of chlorides in water sources. Chloride is also added to water through industrial waste, sewage and sea water. Groundwater has more concentration of chlorides as compared to surface water bodies. Chloride is vital for appropriate functioning in the human body and is not injurious if ingested with suitable amounts of fresh water. WHO recommends maximum permissible limit of 250 mg/l in drinking water.

Potassium is present in both plant and animal cells and is also necessary for all living cells functions. Its deficiency can result in hypertension, irregular heartbeat asthma and kidney diseases. However, its higher intake can also cause irregular heartbeat, kidney diseases, an abnormal protein breakdown, severe infections and weakened immune system. WHO recommends 12 mg/l as a maximum possible of potassium in drinking water.

Sodium is present in natural water either in high or low concentration. It also necessary for the smooth functioning of plant and animal cells. Sodium deficiency in human body can result in low blood pressure, fatigue, dehydration, depression and mental apathy. However, it over dose can also result in hypertension, headaches, stroke, kidney damages, nausea and stomach problems. WHO maximum permissible limit of sodium is 200 mg/l [31].

2.2. Methodology

The Mardan city consists of fourteen Union Councils (UCs). We have selected eight UCs for our study through random sampling tecnique. We have taken sixteen samples of water from private boreholes located within the homes. These samples were collected, on 18th December 2022 using a stratified random sampling technique.

The Polyethylene sterilized bottles were used for the collection of water samples for physical and chemical analysis. After the collection water samples from the study area, we transported them to the Integrated Rural Support Program (IRSP) laboratory in Mardan city for physio-chemical analysis. All the instruments and equipment's were sterilized and calibrated before the analysis of each and every parameter. For the analysis of the physio-chemical parameters of drinking water quality, several instruments and specific procedures were used to measure physical parameters like pH, electrical conductivity, turbidity, alkalinity, total dissolved solids, total suspended solids and chemical parameters like concentrations of ions of potassium, chloride, sodium, nitrate and sulphates. A standardized pH meter was used to measure to pH of collected water samples. For this purpose, each water sample was poured into a clean container. Then the electrode of pH meter was inserted in the water sample until steady reading is recorded. Hardness and alkalinity of each water sample was determined through titration method using methyl orang and phenolphthalein as indicators to show end point of reactions which were related to the hardness and alkalinity levels. Electric conductivity meter, TDS meter and turbidity meter were used to measure electric conductivity, total dissolved solids and turbidity values in the collected water samples in the study area. For chemical analysis of ions (Sodium, potassium, chlorides, sulphates and nitrates) spectrometer was used. For spectrophotometric analysis, reagents specific to each ion type were added, resulted in a color change that were correlated to concentration of ions (Sodium, potassium, chlorides, sulphates and nitrates).

Each of this test was executed with strict quality control to guarantee precise results, permitting laboratory to evaluate whether these water samples meet safe drinking standards or not. Then the data was organized in tabulated form. The data was also plotted on maps using Inverse Distance Weighted interpolation techniques in ArcGIS and spatial distribution maps were prepared. The results obtained were compared with WHO standards and were shown in the form of figures for analysis and interpretation. WHO standard values for these parameters are shown in Table 1 [31].

3. RESULTS AND DISCUSSIONS

3.1. pH

The pH is among the key operational parameter of water quality. The values less than 6.5 pH increase corrosion in water pipes and household sanitation system and greater than 8 is not appropriate for active cleaning. Industrial pollution, Acid rain, and agricultural runoff can change pH level, with bad consequences for both biodiversity and freshwater systems globally. Therefore, the desirable range proposed by WHO is from 6.5 to 8.0. All the samples analyzed had pH values ranges from 6.9 to 7.5. The highest value of pH 7.5 was found in UCs Dagai, Kaskoroona, Muslim Abad and VC Gulibagh I, while lowest values of pH 6.9 were found in UCs Bughdada and Bari Cham respectively. However, all the pH values of sixteen water samples are within permissible limit proposed by WHO. The results of the present investigation are similar to a previous study by Gae *et al*. [32]. The spatial variation in the pH values of sixteen water samples in the study area are shown in Figure 2.

3.2. Total Alkalinity

The total alkalinity values for sixteen water samples are shown in Figure 3. This figure shows that the values of total alkalinity range from 4 to 7 ppm across all samples. The highest alkalinity values

Table 1. Water Quality Standards recommended by WHO [31].

Chemical parameters	Standard (mg/l)	Physical parameters	Standard
Nitrates	50	pH	6.5- 8.5
Sodium	200	Conductivity (µS)	400 µS/cm
Potassium	12	Total Hardness	500 mg/l
Chlorides	250	Total Alkanality as$CaCO_3$	50-500 mg/l
Sulphates	250	Total Suspended Solids	5 mg/l
Fluorides	1.5	Total Dissolved Solids (TDS)	500-1000 mg/l

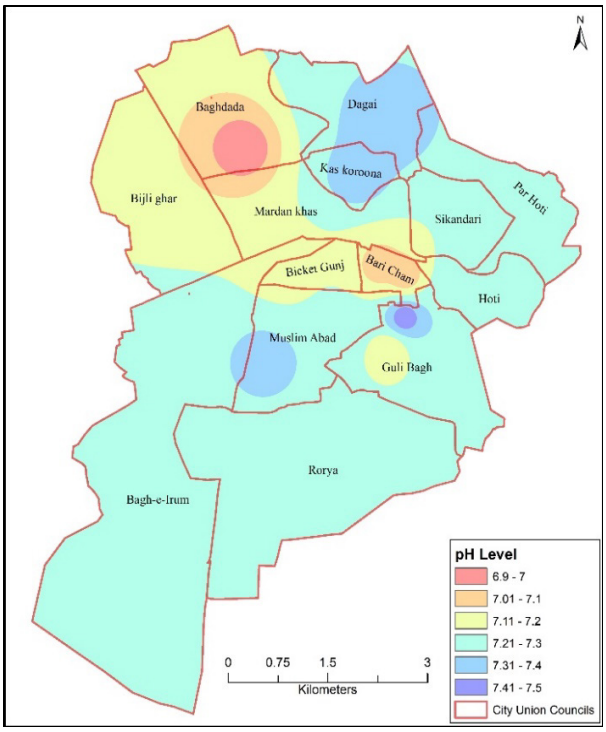

Fig. 2. Spatial distribution of pH.

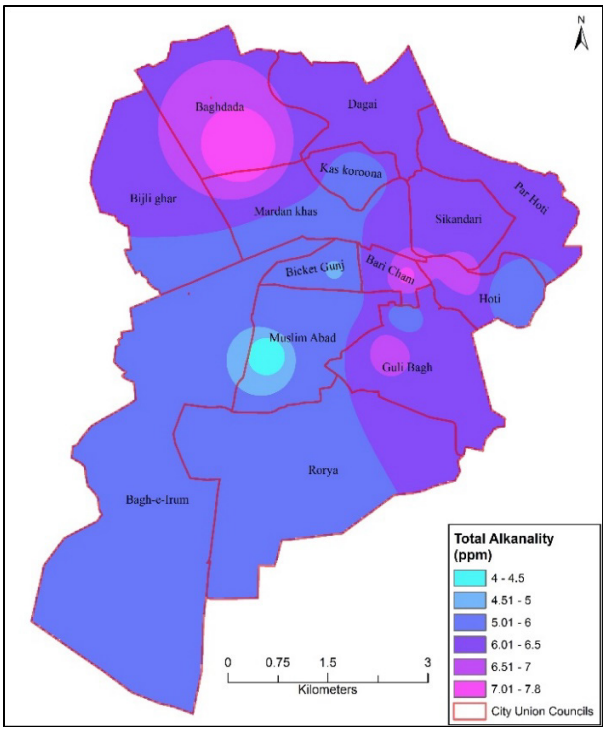

Fig. 3. Spatial distribution of total alkalinity (ppm) in the study area.

were found in the water samples collected from UCs Bughdada, Bari Cham and the lowest alkalinity values were found in UCs Muslim Abad and Bicket Gunj respectively. The maximum permissible level of total alkalinity determined by WHO ranges from 50-500 ppm. It shows that the total alkalinity values of sixteen samples lies within low range determined by WHO. These values of total alkalinity also show low concentration of alkaline salts in these water samples. The geology and the human activities like agriculture and mining can effect the alkalinity in specific geographical area. Alkalinity is important for maintaining stable pH levels in aquatic systems of the world [33]. Hussain *et al*. [5] conducted a research on wtaer quality in Mardan district and found similar results having low alkalinity.

3.3. Total Suspended Solids or Turbidity

The values of turbidity ranges from 0.24 to 0.82 NTU as shown in Figure 4. The highest turbidity values were found in VCs Muslim Abad I and II followed by UCs Bughdada, Dagai and VC Hoti III. While, the lowest turbidity values were found in the water samples collected from VCs Muslim Abad I and Hoti I, respectively. WHO recommended value for turbidity is 0.5 NTU (Table 1). It is evident from Figure 4 that five water samples collected from VCs Hoti I, Muslim Abad I and UCs Baricham, Kaskoroona and Sikandari have values below 0.5 NTU and the remaining eleven samples have values above 0.5 NTU. This shows that the majority of water samples have turbidity values above than the standards recommended by WHO. The higher turbidity values in drinking water increases the chances of gastro-intestinal diseases. The higher turbidity values are especially problematic for the people of weak immunity because the bacteria and viruses can be attached to these solid particles. This process can increase the intensity of gastro-intestinal diseases in those people [34]. Therefore, it is recommended to improve this aspect of water quality in the study area.

3.4. Electrical Conductivity (EC)

The natural water is not a good conductor of electricity. Its electrical conductivity increases with the increase of contamination in water. The recommended value for EC is 400 µS/cm for drinking water recommended by WHO (Table 1). However, its permissible limit is 800 µS/cm for human consumption. The electric conductivity values of all sixteen water samples ranges from 700 to 1480 µS/cm. The highest values of EC were found in the water samples collected from UCs

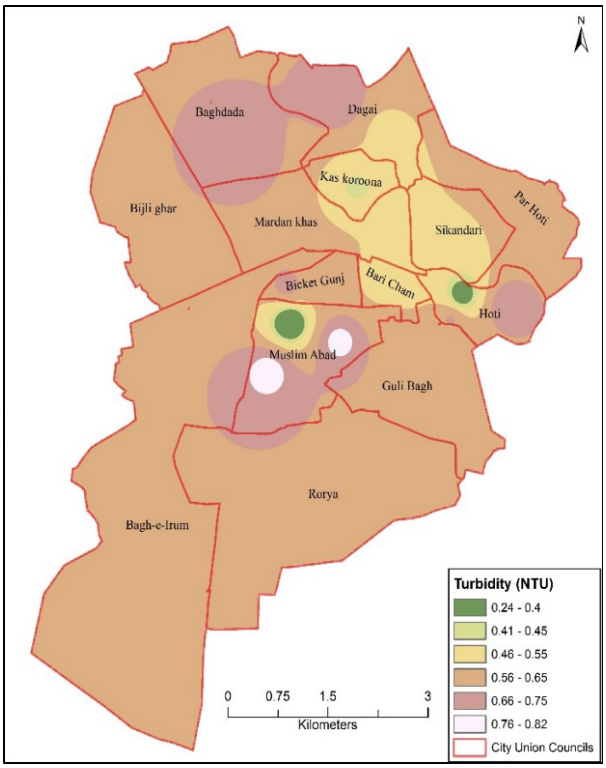

Fig. 4. Spatial distribution of turbidity (NTU).

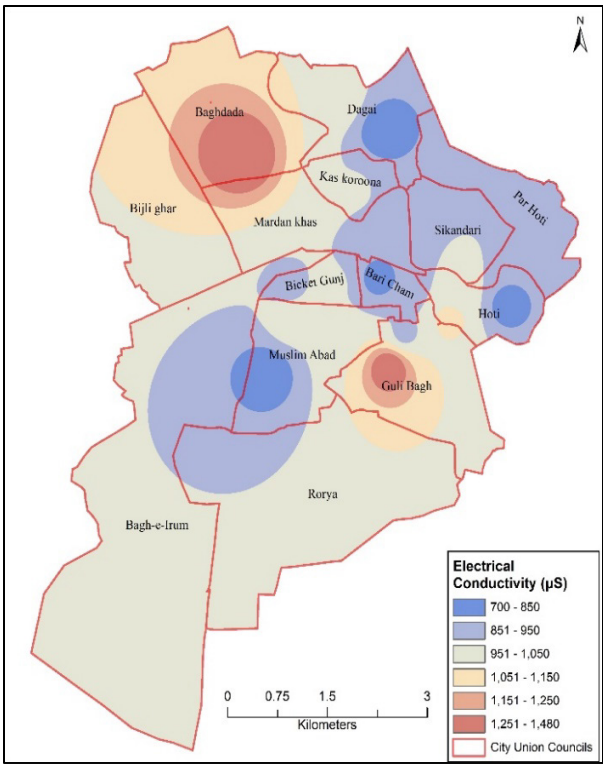

Fig. 5. Spatial distribution of electric conductivity.

Bughdada and Gulibagh. While the lowest values of EC were found in water samples collected from UCs Dagai, Baricham, Hoti and muslim as shown in Figure 5. It is evident from these results that the majority of water samples have higher values than permissible limit prescribed by WHO. High conductivity values in drinking water has no direct health impacts. However, indirectly it can increase alkalanity or hardness in drinking water that may effect consumer satisfiction [35].

3.5. Total Dissolved Solids

Total dissolved salts spatial distribution is shown in Figure 6. It is ranging from 406- 841 mg/l. The highest values of dissolved salts are found in UCs Bughdada and Gulibagh and the lowest values are found in UCs Dagai, Hoti and Muslim Abad (Figure 6). The WHO recommends 500 mg/l as a permissible limit of total dissolved solids and 1000 mg/l as a admissible limit for drinking water. This indicates that all the sixteen water samples are within the permissible and admissible limit proposed by WHO. Total dissolved solids excessive amounts are unpleasant in drinking water because of the potential physiological influence, unfriendly mineral taste and decay [36].

3.6. Total Hardness

The values of total hardness as a $CaCO_3$ of sixteen private bores samples are shown in figure 7. These values range from 30-400 mg/l which are within the permissible limit of 500 mg/l recommended by WHO [31]. The highest values of hardness were found in the UCs of Bugdada, Baricham and Hoti and the lowest values were found in UC Muslim Abad as shown in Figure 7. All these values of hardness are still higher than desirable value of 100 mg/l of WHO except a single sample whose value is less than 100 mg/l. However, this guideline value is not given on the health basis. Human beings can afford hardness greater than 500mg/l. However, higher values of hardness can create problems for kidney and heart patients. It is therefore, necessary to control factors contributing hardness in water. A research study by Mustafa *et al*. [37] on physio-chemical and biological analysis of drinking water quality from the residential areas of Islamabad, Pakistan have also reported similar results in which all selected water samples have less than 500 mg/l values of total hardness and were also within permissible limit of WHO.

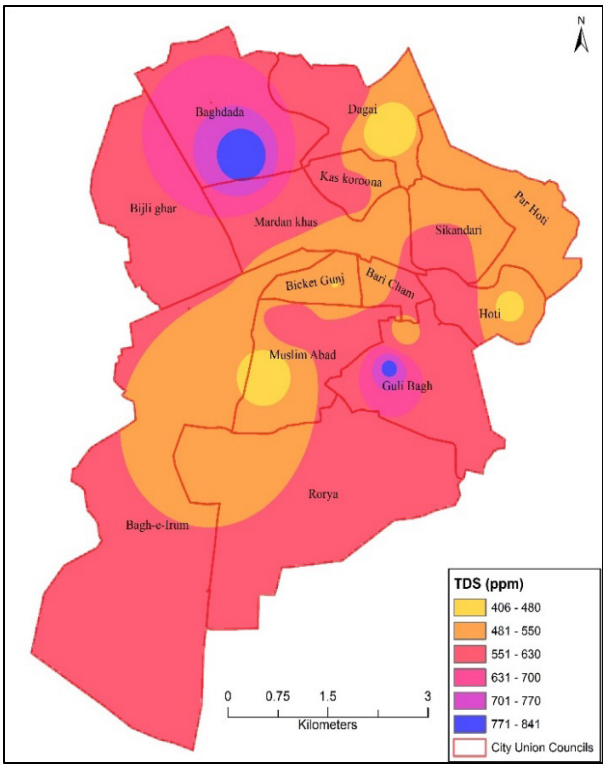

Fig. 6. Spatial distribution of total dissolved solids.

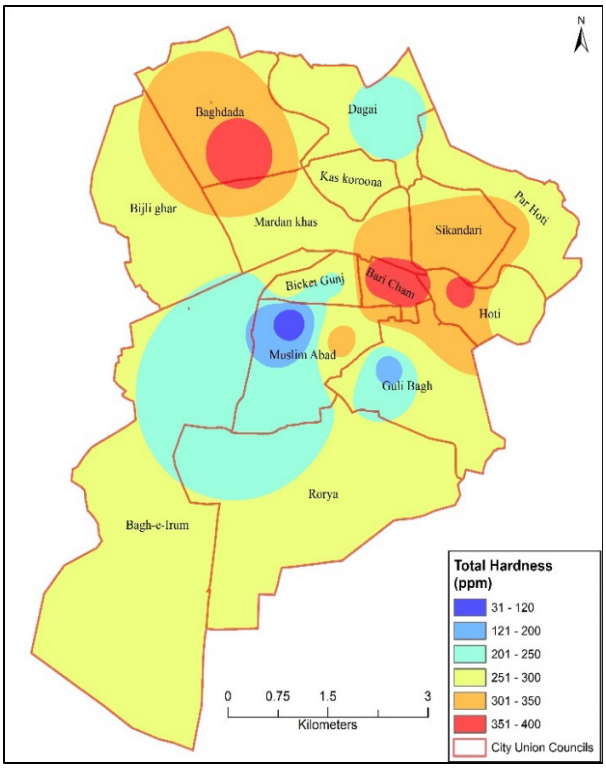

Fig. 7. Spatial distribution of total hardness (ppm).

3.7. Potassium (K^+)

The concentration of potassium in water samples varies from 3.7 to 14.3 ppm (Figure 8). The highest potassium concentration were found in the UCs Hoti, Bicket Gunj and Bughdada while the lowest values of potassium were found in UCs Dagai, Hoti, Bari Cham and Muslim Abad, respectively, as shown in Figure 8. WHO recommends 12 ppm as a maximum possible of potassium in drinking water. All the water samples except a single sample at UC Hoti has potassium concentration within the limit recommended by WHO. It shows that the majority of the water samples areas are safe for drinking purpose as far as potassium concentration in drinking water is concerned. Potassium is present in both plant and animal cells and is also necessary for all living cells functions. Its deficiency can result in hypertension, irregular heartbeat asthma and kidney diseases. However, its higher intake can also cause irregular heartbeat, kidney diseases, an abnormal protein breakdown, severe infections and weakened immune system [38].

3.8. Nitrates (NO_3)

The values of nitrate for sixteen water samples are in the rangeof 3.6 to 7.1 ppm. The highest concentrations of nitrate ions were found in the water samples collected from UCs Bughdada, Bicket Gunj and VC Muslim Abad II and lowest concentration of niterates in UCs Dagai, Kaskoroona, Hoti and VC Muslim Abad I as shown in Figure 9. The minimum value of nitrate is 40 ppm and the maximum value is 70 ppm which are within the permissible level as recommended by WHO. Similar low concentration of niterates were reported by another research study conducted on drinking water quality in district Mardan [25].

3.9. Sulphates

The concentration of sulphates in water samples is shown in Figure 10 indicating a range from 35-511ppm while the highest permissible limit of sulphates recommended by WHO is 250 ppm for drinking water. The highest concentration of sulphates was found in the water sample collected from VC Gulibagh I and the lowest concentration of sulphates ions were found in UCs Dagai, Hoti, Muslim Abad and Bicket Gunj respectively. The Figure 10 shows that all water samples have sulphates concentration within permissible limit except sample collected from VC Gulibagh I whose

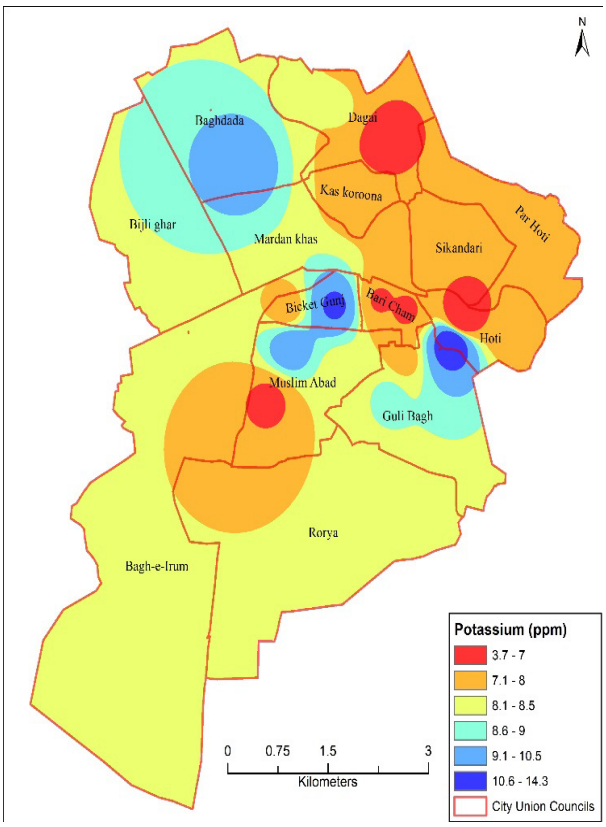

Fig. 8. Spatial distribution of potassium (ppm).

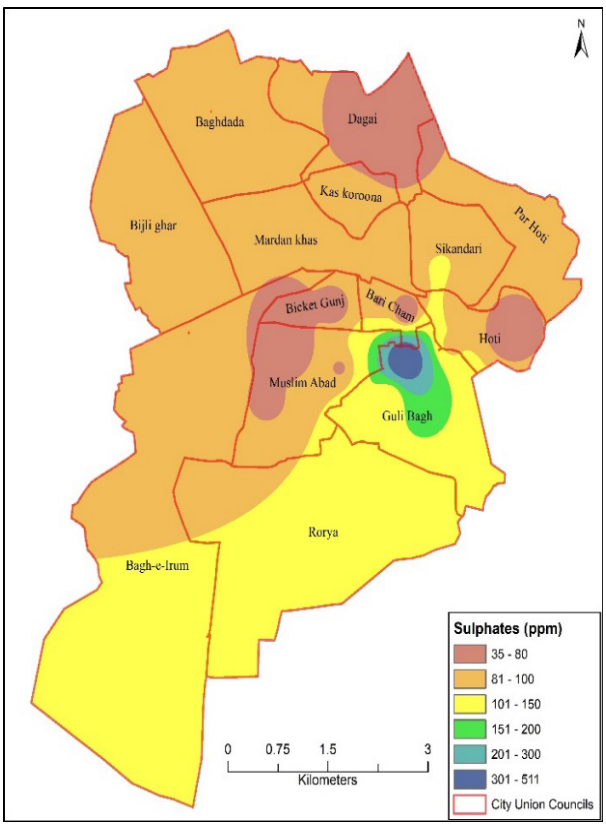

Fig. 10. Spatial distribution of sulphates (ppm).

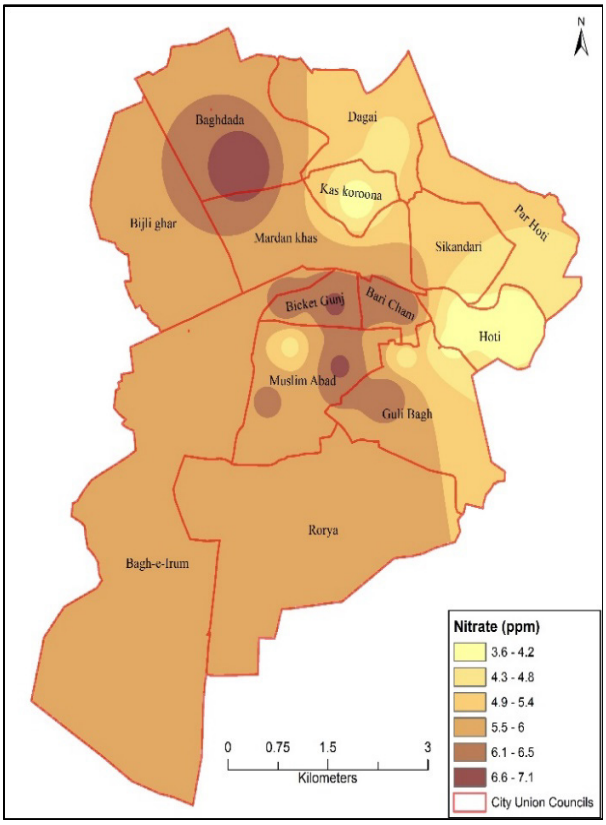

Fig. 9. Spatial distribution of nitrates (ppm).

concentration is far greater than recommended limit of WHO. High concentration of sulphates in drinking water can result in gastro-intestinal irritation and catharsis. Its higher concentration can also affect the taste of drinking water. Leaching of sulphate fertilizers to the water table is one of the major causes of high concentration sulphates in drinking water. Sulphates are present in almost all natural water due high solubility nature of water. A reseach on the physio-chemical analysis of drinking water was carried out in district Dir, Khyber Pakhtunkhwa, Pakistan by Shakirullah *et al*. [39]. The results of that study also revealed high concntration of sulphates in two drinking water samples and the remaining water samples have the sulphates ions concentration within the permissible limit of WHO.

3.10. Chlorides

The Figure 11 shows the concentration of chlorides in water samples which ranges from 25-170 mg/l and are found within the range of WHO limits as the maximum recommended chlorides limit is 250 ppm for portable drinking water (Table 1). The highest

value of chloride ions were found in the water samples collected from UC Bughdada, Gulibagh and VC Muslim Abad I and the lowest values of chloride were reported in UC Hoti, Baricham and VC Gulibagh I respectively. It is evident from the figure 11 shows that all sixteen samples of water has chlorides concentration within permissible limit of WHO. These results are compareable to a study conducted on physio-chemical analysis of drinking in district Dir, where it was found thatchlorides ions concentration were within permissible limits of WHO [39]. It can be concluded that these water samples are safe for human drinking purpose as far as chlorides concentration is concerned.

3.11. Sodium (Na^+)

Sodium is present in natural water either in high or low concentration. It also necessary for the smooth functioning of plant and animal cells. Sodium deficiency in human body can result in low blood pressure, fatigue, dehydration, depression and mental apathy. However, it over dose can also result in hypertension, headaches, stroke, kidney damages, nausea and stomach problems. The values sodium in sixteen water samples is shown in Figure 12. The figure 12 shows that the values of sodium in these water samples range 60-170 ppm. The highest values of sodium ions were found in the water samples collected from UCs Bughdada and Gulibagh and the lowest values of sodium were found in UCs Hoti, Bari Cham and Muslim Abad as evident from figure 12. WHO maximum permissible limit of sodium is 200 ppm. It shows that the sodium concentration in all the water samples is within the permissible limit of WHO and is safe for drinking purposes. A study conducted by Arega [40] on sodium and potassium analysis of drinking water quality assessment and its health effects in Ethiopia found that majority of water samples have less than 200 mg/l of sodium concentration.

4. CONCLUSIONS

The physical parameters (pH, alkalinity, total hardness, and total dissolved solids) and chemical parameters (sodium, chloride and nitrate) in all sampled areas fall within the WHO recommended limits for drinking water quality. However, this study identified abnormalities in some specific

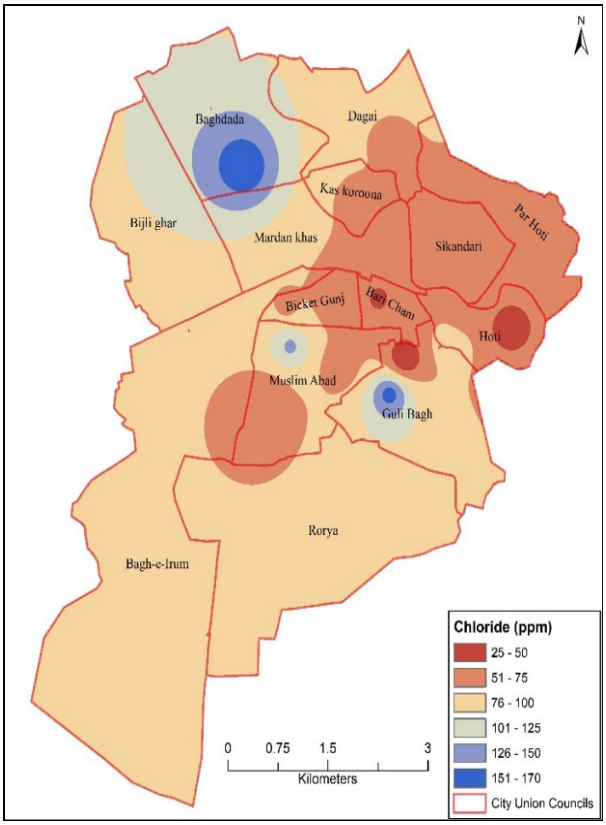

Fig. 11. Spatial distribution of chloride (ppm).

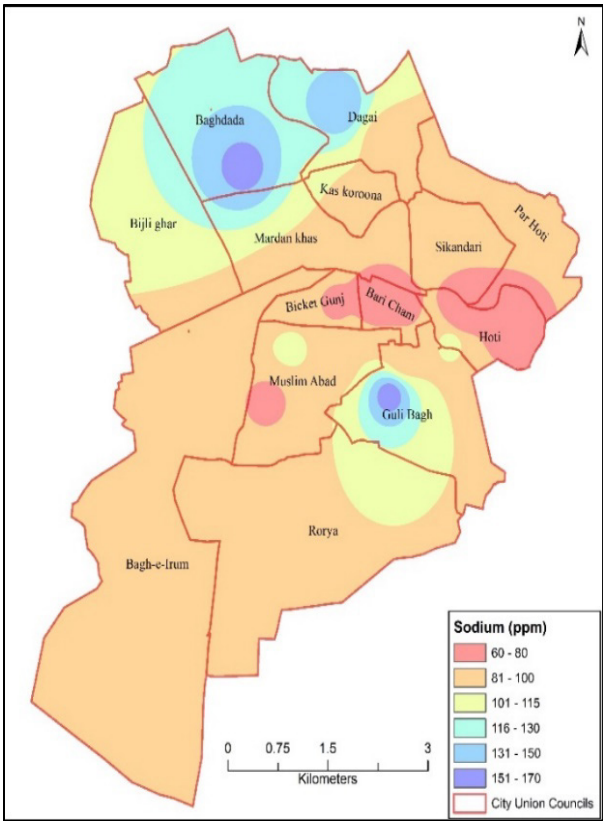

Fig. 12. Spatial distribution of sodium (ppm).

locations regarding physical parameters (turbidity 0.82 NTU and electrical conductivity 1480 µS/cm) and chemical parameters (potassium 13.7 mg/l and sulphates 512 mg/l). These values exceed the WHO guideline limits for drinking water. Therefore, it can be concluded that the quality of groundwater at majority places in the study are safe for drinking purpose. However, it is recommended that the concerned department should implement effective measures to ensure the provision of safe drinking water in accordance with WHO standards for all residents.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declared that they have no competing interests.

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Research Article

Effects of Plant Growth Promoting Bacteria on Growth and Essential Oil Production of Peppermint

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Abstract: Peppermint is abundant with organic compounds having therapeutic, coloring, preservative, and other uses for humans. Moreover, peppermint is used in food and perfume industry and as medicine in every corner over the world. Considering the economic and medicinal value of peppermint, there is a significant gap between supply and demand. Therefore, an analysis of the impact of bacteria that promote plant development was carried out on peppermints (*Pseudomonas putida* and *Curtobacterium* sp. *strain* LUW) essential oil production. Under the study, the plant growth parameters, essential oil, chlorophyll and carotenoids, and free proline of leaves were measured. The outcomes demonstrated that the use of both *Pseudomonas* and *Curtobacterium* (Plant Growth Promoting Bacteria) in the soil significantly increased root diameter, leaf area, leaf number, plant height, quantity of stems, root length, root volume, dry weight of leaf, and peppermint plants' relative water content by 34.29, 23.34, 36.57, 21.08, 87.5, 12.28, 20.37, 42.62, and 6.46 compared to the control conditions respectively. Furthermore, the application of bacteria in the soil that promotes plant growth raised the total quantity of essential oil, proline, and chlorophyll concentration in the leaf by 50.90, 20.90, and 33.35%, respectively, compared to the control conditions. Moreover, essential oil and proline content increased with *Curtobacterium* sp. *strain* application compared to *Pseudomonas putida.*

Keywords: *Curtobacterium* sp. *strain*, Essential Oil, Growth, Peppermint, *Pseudomonas putida*.

1. INTRODUCTION

It is well known that medicinal plants are abundant with chemicals having therapeutic, coloring, preservative, and other uses for human beings [1]. Peppermint (*Mentha piperita* L.) a perennial herbaceous plant, related to the family of Lamiaceae, genera *Mentha*, used as a medicine and immune-boosting food all over the world [2]. Water mint (*M. aqutica*) and spearmint (*M. spicata*) naturally crossed to produce peppermint [3]. Peppermint has a rhizome and short root, and the height of plant reaches about 30 to 90 cm. Peppermint has a straight, ascending, and branched stem, which is completely square in the upper part and it is reddish-purple or purple in color, and has two opposite leaves in each of the nodes. This plant has ovate leaves elongated to lance-shaped, petiolate, pointed toothed, slightly covered with hair, and opposite, 4 – 9 cm in length and 1.5 – 4 cm in width, whose upper surface is dark green. Peppermint has irregular flowers, mostly bisexual or hermaphrodite, having red, purple, or white flowers depending on the type of peppermint, and appear in the months of August and September. Its fruits are oval and hazelnut-shaped [4]. Approximately, all essential oils are synthesized and stored in the secretive hairs located on the surface of leaves [5]. Essential oil of peppermint has different contents such as menthyl acetate and menthone (monoterpenea), menthol (cyclic monoterpene alcohol), and a small amount of cineole and other terpenes.

Plant growth-promoting bacteria (PGPBs) include *Azospirillum*, *Azotobacter*, *Rhodococcus, Bacillus*, and *Pseudomonas* produce hormone-like substances, reduce ethylene levels, prepare and absorb plant-required elements such as

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phosphorus, iron, nitrogen, and potassium, and produce antimicrobial compounds which increase the host plants' ability to withstand a variety of environmental stresses including pests, diseases, salinity and drought [6]. *Azotobacter* is very important in the biological fixation of nitrogen and *Bacillus* and *Pseudomonas* in the transformation of phosphorus from its insoluble forms into soluble forms that plants can absorb. In addition, by generating siderophores for example, these bacteria promote plant growth in several ways, synthesis of antibiotics, and production of plant hormones [7]. Benchio *et al*. [8] reported that the application of *Bacillus* growth-promoting bacteria to basil plants resulted in a twofold increase in the amount of plant essential oil as well as an increase in biomass.

Considering the economic importance of peppermint plant in various pharmaceutical and food industries, this study aimed to examine the impact of *Pseudomonas putida* and *Curtobacterium* sp. *strain* LUW, two plant growth-promoting bacteria, on the development and yield of peppermint essential oil.

2. MATERIALS AND METHODS

This study was conducted at the research farm of Lorestan University, Iran, in 2022 (in greenhouse) located 33.38^{o} north and 48.35^{o} east, 1147 m above sea level, and an average temperature of 25±5 °C during the growing season. Rhizomes (*M. piperita* L.) were acquired from Research and Technology Complex of Medicinal Plants, Lorestan Province. Iran, and bacterial strains of *Curtobacterum* sp. *strain* LUW and *Pseudomonas putida* from the bacteriological collection from the Department of Plant Protection at the Agriculture Faculty, Lorestan University, Lorestan, Iran. Before planting the rhizomes, *Curtobacterium* and *Pseudomonas* bacteria were placed in separate pots between the pots filled with water, later peppermint rhizomes were placed between the pots containing the bacteria to inoculate the roots for a period of time. It was left in the pan for an hour. The strains were cultured in Vitro. Three treatments, i.e., control, *Pseudomonas putida and Curtobacterum* sp. *Strain* each with 8 replications were used. The rhizomes of peppermint were subjected to inoculation with two species of PGPBs. For this purpose, utilizing a fully randomized design, a factorial experiment was performed. Thereafter, the peppermint roots were inoculated with plant growth-stimulating bacteria, the inoculated rhizomes were grown in pots containing two kilograms of sterilized agricultural soil, manure, and cocopeat in a 2:1:1 ratio. The morphological traits including plant height, plant crown, number of leaves, branches and internode, length, volume and dry weight of root, leaf dry weight, length of internode, fresh and dry weight of stem were measured. The chlorophyll and carotenoids content were measured using Lichtenthaler method [9], free proline in leaves was measured using Bates method [10], and essential oil from dried leaves and petioles was measured using the distillation method [11]. The data were analyzed using Fisher LSD method and 95% confidence.

3. RESULTS AND DISCUSSION

3.1. Morphological Features

Variance analysis of data showed that *Pseudomonas putida* and *Curtobacterium* sp. *strain* LUW plant growth-promoting bacteria increased plant growth parameters such as plant height, leaves, leaf area, crown diameter, number of stems, roots' diameter, length, and volume, internode number, length of internode, weight of fresh and dry leaf, weight of fresh and dry stem, weight of fresh and dry root, and relative water content by 21.08, 36.57, 23.35, 31.13, 87.5, 34.29, 12.28, 20.37, 13.79, 18.54, 28.18, 42.62, 39.11, 52.20, 49.08, 40.06, and 6.460% respectively, in comparison to control. The results indicated that the application of PGPBs increased morphological characteristics such as plant height, quantity, and leaf surface, crown diameter, quantity of stems, diameter, volume, and length of the roots, number and length of internodes significantly. Which shows the positive effects of plant growth promoting bacteria about peppermint's development and growth (Table 1).

According to Ferreira *et al*. [11], the increase in the growth is because of nitrogen fixation, dissolution of mineral phosphates, iron absorption with siderophore, adjusting the level of plant hormones such as auxin or ethylene (through ACC (1-Aminocyclopropane-1-Carboxylate) deaminase activity), and combating pathogens by releasing hydrogen cyanide. Other studies' findings demonstrated that the use of PGPBs increases plant height, diameter of the stem, leaf count, length and quantity of branches, and dry and fresh weight of aerial parts [12]. Studies on black seed [13], sage

Table 1: Effect of PGPBs application on the morphological traits of peppermint.

Plant growth stimulating bacteria	Plant height (cm)	Leaves (number)	Shoots (number)	Root volume (cm^3)	Internode (number)	Dry weight of leaf (g)	Dry weight of stem (g)	Dry weight of root (g)
Control	22.50 b	45.58 b	5.41 b	10.75 b	6.58 b	1.15 b	0.83 b	1.43 c
Pseudomonas putida	28.33 a**	67.33 a**	6.75 a**	12.95 a**	7.33 ab**	1.39 a**	1.20 a**	1.91 b**
Curtobacterium strain	27.91 a**	65.41 a**	6.25 ab**	12.16 a**	7.58 a**	1.39 a**	1.20 a**	2.16 a**

** and * are significant at the level of 1 and 5% probability.

plant [14], and pumpkin [15], also figured out that the application of PGPBs increases the growth of plants. Numerous researches indicated that the increase in plant growth is due to PGPBs induction of growth-promoting hormones such as auxins and gibberellins, which increase the number and length of plant cells. By changing the structure of the root system, PGPBs enhance nutrient absorption, allocation of carbohydrates to the root, reduction of root peroxidase activity, synthesis of new proteins, and ultimately cause a rise in the growth of plant roots [16].

In the present study, the use of PGPBs increased the growth coefficient in peppermint plants. Thus, the increase and improvement in growth were perhaps because of auxin and gibberellin production and absorption of nutrients like nitrogen, phosphorus, potassium, magnesium, and boron. Several other researches also reported the positive influence of growth-stimulating bacteria on the height of various plants [17]. Similar results having positive effect of PGPBs on the increase of the total number of leaves was reported by Fasihi *et al*. [18], and the total number of branches by Yasri *et al*. [19].

3.2. Essential Oil

Application of *Pseudomonas putida* and *Curtobacterium* sp. *strain* LUW (PGPBs) increased the percentage of plant essential oil by 34.61 and 50.90% respectively, compared to the control. The amount of peppermint essential oil significantly increased when PGPBs were used. Leithy *et al*. [20] reported that the use of *Azotobacter*, *Azospirillium,* and phosphate-dissolving bacteria increased essential oil production in *Mazorana hortensis*. According to Singh *et al*. [21] essential oil is increased in leaves by using Nitrogen (*Mentha arvensis* and *Mentha piperita*). Benchio *et al*. [8] also stated that the utilization of Bacillus plant growth stimulating bacteria on the basil plant raised biomass and the amount of basil essential oil by two times. Rati *et al*. [22], concluded that the application of phosphate-dissolving bacteria together with insoluble inorganic phosphate called tricalcium phosphate caused a significant improvement in the concentration of phosphorus in the stem and a notable rise in essential oil percentage compared to control.

3.3. Chlorophyll and Carotenoids

Inoculation of peppermint rhizomes and soil with *Curtobacterium strain* and *Pseudomonas putida* plant growth-promoting bacteria, increased the amount of chlorophyll in the whole leaf by 33.35% (chlorophyll-a 37.91% and chlorophyll-b by 26.10%) and 25.15% (chlorophyll-a 33.56% and chlorophyll-b 13.72%) in contrast to the treatments under control. The content of carotenoids in the plant leafs increased by 49.98% and 56.88% in contrast to the control with the application of *Pseudomonas putida* and *Curtobacterium strain* plant growth-promoting bacteria, respectively. However, the differences between *Pseudomonas* and *Curtobacterium* bacteria were not statistically significant (Table 2).

The rise in the amount of chlorophyll with growth-promoting bacteria may result from the remarkable superiority in producing siderophores in these above-mentioned bacteria. Additionally, auxin is important in raising the amount of chlorophyll, so in an experiment, the treatment of wheat leaves with 100 mg/kg indole-3-acetic acid caused a 31% increase in the chlorophyll content in contrast to the treatment under control [17]. The research conducted by Iftikhar *et al*. [23] suggested

Table 2. Effect of PGPBs application on the production of essential oil, chlorophyll carotenoid, and leaf proline.

Plant growth-promoting bacteria	Essential oil %	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid	Leaf proline
Control	0.39 b	6.26 c	4.60 b	10.87 c	1.37 b	0.43 a
Pseudomonas putida	0.53 a**	8.36 b**	5.23 a**	13.60 b**	2.20 a**	0.33 b**
Curtobacterium strain	0.60 a**	9.45 a**	5.82 a**	15.28 a**	2.15 a**	0.39 a**

** and * are significant at the level of 1 and 5% probability.

that PGPBs with the ability to dissolve phosphate by producing acidic compounds not only can increase the phosphorus's solubility, but additionally boost the uptake of magnesium and iron, which are essential elements for the production of chlorophyll.

3.4. Proline

Inoculation of peppermint rhizomes and soil with *Pseudomonas putida* and *Curtobacterium sp.* PGPBs enhanced the concentration of plant proline by 20.90% compared to the control treatments. Applying PGPBs effectively increased the proline content of leaves (Table 2). Proline is produced by the nitrogen metabolism of plants, where nitrate is first transformed into nitrite and then ammonia, following which glutamine and glutamate are used to convert it to amino acids. Therefore, PGPBs contribute to the above process by fixing nitrogen and increasing the plant's proline. The results of the study show that PGPBs can fix nitrogen. Consequently, the increase in proline in peppermint can be linked to the peppermint plant's bacteria producing more nitrogen [18].

4. CONCLUSIONS

According to the Findings of the study, application of both *Pseudomonas* and *Curtobacterium* (PGPBs) in the soil significantly enhanced plant height, leaf area, number of leaves, root diameter, number of stems, root length, root volume, dry leaf weight, and relative water content of peppermint plants in comparison to the control circumstances. Furthermore, applying of plant growth stimulating bacteria in the soil increased the total amount of essential oil, Carotenoid, and chlorophyll content in the leaf.

5. CONFLICT OF INTEREST

The authors declare that there is conflict of interest.

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Research Article

Effect of Substituting Table Sugar with Date Fruit Powder on the Nutritional and Sensorial Properties of Cake

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Abstract: Despite the World Health Organization's advice to limit sugar consumption, consumers unwillingly rely on table, refined, or added sugars owing to a lack of suitable alternatives. Date powder (DP) is a potential nutraceutical reservoir that can be an excellent natural sugar or table sugar (TS) replacer. Since *Dhaki* is one premium date fruit variety, it was utilized in its *Tamer* stage for developing DP owing to its higher sugar content, shelf-life stability, and low moisture. The date fruit was cleaned, depitted, thermally dehydrated (50±1°C for 2 days), powdered, and utilized as a TS replacer in cakes. A total of four formulations of cake sweeteners were set (i.e., T_0 = control/ cake without DP, T_1 = 10% DP + 90% TS, T_2 = 20% DP + 80% TS, T_3 = 30% DP + 70% TS). DP and cake formulations were subjected to assessment as per standard analytical methods. Findings showed that DP had 3.45% moisture, 5.22% protein, 0.78% fat, 6.84% fiber, 5.23% ash, 21.34% carbohydrate, 68.18% reducing sugar, 3.33% non-reducing sugar and 71.51% total sugar with pH value 5.4. The nutritional and sensorial properties of all cake samples (T_0-T_3) remained statistically different ($p < 0.05$). Among all samples T_3 had significantly higher ($p < 0.05$) average values for fat (19.03%), protein (15.44%), ash (5.77%), carbohydrate (61.36%), fiber (3.51%), non-reducing sugar (6.85%), energy value (478.47 kcal/100g), color (8.33), texture (8.33), taste (9.0), aroma (8.33), and overall acceptability (8.50) followed by T_2, T_1, and T_0. Findings conclude that increasing DP proportion in cakes not merely improved sweetness but also enhanced their nutritional value (fiber content, energy value, etc.), and sensorial attributes. DP is therefore recommended to be used as one suitable TS replacer in a variety of food products.

Keywords: Date Fruit Powder, Table Sugar Replacer, Baked Goods, Nutritional Assessment, Sensorial Properties.

1. INTRODUCTION

Date (*Phoenix dactylifera* L.) being a significant crop of the *Arecaceae* family has been cultivated for over 4,000 years in many arid regions of the Middle East and Northern Africa for date fruit [1, 2]. Pakistan ranks fifth in the world among date fruit-producing countries while date fruit is the country's fourth most popular fruit [3]. This fruit is versatile since having immense nutritional, medicinal, nutraceutical, etc. attributes [4]. It contains high quantities of sugars (fructose and glucose), dietary fibers, micronutrients, etc., [5]. Date fruit sugars have a low glycemic index and can be absorbed more promptly in blood and tissues than refined sugars [6]. An excessive intake of refined or table sugar (TS) has been recognized as a crucial risk factor for type 2 diabetes, obesity, cancer, cardiovascular diseases, etc. [7]. Despite this fact, the consumption rate of table sugar is quite high in most Asian countries. Researchers are therefore exploring appropriate interventions to substitute TS with fruit-based natural sugar for acquiring a sweet taste in foods to limit consumers' health risks. A substantial review of scientific literature suggests the adequacy of using date fruit as a TS replacer [8-11]. In Pakistan, the provinces of Baluchistan and Sindh are major contributors to date fruit production

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with leading varieties, i.e., *Mozawati, Aseel, Dhaki, Zahidi, Fasli,* etc., [1, 12]. The date fruit has four maturity stages, it achieves firm texture, dark brown color, and maximum sweetness in *the Tamer* stage (last stage of maturation) hence is the ultimate fruit stage to be utilized for developing date powder (DP). Date fruit in various forms may serve as a suitable TS replacer (i.e., date syrup, paste, and powder). Since, among various baked foods, cakes acquire a premium place due to their sweetness and richness of taste. In the present study, DP was developed from var. *Dhaki* and utilized against table sugar as a TS replacer in cakes. However, the nutritional and sensory quality characteristics of the cake samples were also determined to assess their suitability.

2. MATERIALS AND METHODS

2.1. Collection of Raw Materials

Date fruit (var. *Dhaki*) was purchased from the National Super Mart near Hala Naka, Hyderabad City, Pakistan. Raw materials for preparing cake samples (i.e., fine wheat flour, butter, table sugar, baking powder, fresh eggs, etc. were procured from a grocery store in Hyderabad City, Pakistan.

2.2. Preparation of Raw Materials

2.2.1. Drying of date fruit/ development of date powder

Date powder was prepared as per the method described by Manickavasagan *et al.* [13] with slight modifications. The date fruits were cleaned/sorted manually to remove any bruised or unhealthy fruit from the entire lot. The selected date fruits were properly washed with running tap water to remove dust and debris, air dried at room temperature, de-pitted manually using a stainless-steel knife, sliced (0.5 cm thickness), and subsequently set on to the perforated trays of cabinet dehydrator at 50 ± 1°C for 2 days. After drying, dried date fruit slices were milled in an electric mill to obtain date powder. Finally, the date powder was sieved through 80 mesh sieve and kept in an air-tight glass jar till further use.

2.2.2. Development of cake samples

The method described by Ghasemi *et al.* [14] was followed for developing date powder-enriched cake samples with some minor modifications. The batter of cake was prepared using ingredients such as fine wheat flour, butter, table sugar/icing sugar, date fruit powder, baking powder, milk powder, eggs, etc., (the detailed ingredients used for making cake samples are given in Table 1). The fine wheat flour and icing sugar were sifted, eggs were beaten, and other ingredients to be used were weighed. Later, all ingredients were taken in a bowl mixed properly using an electric beater, poured into the mold, and baked in an electric oven for about 25 minutes at 180 ºC. After baking, the cake samples were removed from the oven and cooled at room temperature. The cakes were sliced (1.5 cm), packed into the box, properly labeled, and used for sensory evaluation from panelists and chemical analysis.

Table 1. List of ingredients used for making cake samples.

Ingredients	Treatments of cakes			
	Control/T_0	T_1	T_2	T_3
Fine wheat flour (g)	100	100	100	100
Date powder (g)	-	8	16	24
Table sugar (g)	80	72	64	56
Egg whole	1	1	1	1
Milk powder (g)	10	10	10	10
Baking powder (g)	1	1	1	1
Butter (g)	20	20	20	20
Fat (g)	20	20	20	20
Salt (g)	1	1	1	1
Vanilla essence (drops)	2	2	2	2

2.3. Sample Analysis

2.3.1. Nutritional assessment

The pH value of date powder and cake samples was determined by using a digital pH meter (Model HI, Hanna Instruments, Italy) according to the method of AOAC [15]. By following the standard methods of AOAC [16], i.e., moisture (%), fat (%), protein (%), ash (%), carbohydrate (%), and fiber (%) were analyzed. The energy value (kcal/100g) of date powder and cake samples was calculated as per the method by Paul and Southgate [17]. The method described by Awan [18] was used to examine the reducing sugar (%) and total sugar (%) of date powder and cake samples. Non-reducing sugar (%) was determined as per the difference method by using the formula given below:

$$\text{Non} - \text{reducing sugar (\%)} = \text{Total sugar} - \text{Reducing sugar}$$

2.3.2. Determination of sensorial attributes

For descriptive sensory evaluation, a group of 10 trained panelists with 50:50 (male: female) gender ratio, aged 22-56 years was requested to participate in the study by taking formal consent for sensory evaluation of cake samples. The sensorial attributes, i.e., color, texture, taste, aroma, and overall acceptability of cake samples were determined as per the method described by Iwe [19] using nine-point hedonic scale (i.e., 9 = like extremely to 1 = dislike extremely).

2.4. Statistical Analysis

A total of three replications were studied during the studies for all tests (nutritional and sensorial properties). The data obtained from the present study was tabulated in Excel and analyzed using Statistical Package for the Social Sciences-20 for one-way ANOVA. The mean values range (maximum and minimum) at p-value < 0.05 were evaluated.

3. RESULTS

During the study, freshly developed date fruit powder was evaluated for nutritional analysis. The results regarding the nutritional and sensorial properties of all cake treatments are presented in this section.

3.1. Nutritional Analysis of Date Fruit Powder

The results regarding the nutritional analysis of date fruit powder are presented in Table 2. The result revealed that date fruit powder showed to have 3.45% moisture, 5.22% protein, 0.78% fat, 6.84% fiber, 5.23% ash, 21.34% carbohydrate, 68.18% reducing sugar, 3.33% non-reducing sugar, and 71.51% total sugar while pH value remained 5.4.

3.2. Nutritional Attributes of Cake Samples with Different Levels of Table Sugar and Date Fruit Powder

The results regarding the average nutritional attributes of cake samples prepared with different levels of sugar and date powder are presented in Tables 3 and 4. The observed results revealed that all treatments showed statistically different ($p<0.05$) mean values.

The moisture content in cake samples varied from 17.61% to 23.12%, with T_0 showing the highest moisture content (23.12%), followed by T_1 (20.34%), T_2 (19.03%), and T_3 (17.61%). The pH values ranged from 5.67 to 6.94, with T_3 having the highest pH (6.94) followed by T_2 (6.33), T_1 (5.81), and T_0 (5.67). Fat content ranged from 17.23% to 19.03%, with T_3 showing the highest fat content (19.03%), followed by T_2 (18.88%), T_1 (18.07%), and T_0 (17.23%). Protein content ranged from 12.03% to 15.44%, with T_3 having the highest protein content (15.44%), followed by T_2 (14.54%), T_1 (13.92%), and T_0 (12.03%). Ash content varied from 1.89% to 5.77%, with T_3 showing the highest

Table 2. Nutritional attributes of date fruit powder.

Nutritional attributes	Average values
Moisture (%)	3.45
Protein (%)	5.22
Fat (%)	0.78
Fiber (%)	6.84
Ash (%)	5.23
Carbohydrate (%)	21.34
Reducing sugar (%)	68.18
Non-reducing sugar (%)	3.33
Total sugar (%)	71.51
pH value	5.4

Table 3. Nutritional attributes of cake samples with different levels of sugar and date powder.

Treatments	Moisture %	pH Value	Fat %	Protein %	Ash %	Carbohydrate %
T_0	23.12 a	5.67 d	17.23 d	12.03 d	1.89 d	56.18 c
T_1	20.34 b	5.81 c	18.07 c	13.92 c	3.89 c	58.49 b
T_2	19.03 bc	6.33 b	18.88 b	14.54 b	4.56 b	59.98 ab
T_3	17.61 c	6.94 a	19.03 a	15.44 a	5.77 a	61.36 a
SE ±	0.8186	0.0312	0.0216	0.0286	0.0252	0.7049
LSD (0.05)	1.8877	0.0719	0.0498	0.0659	0.0580	1.6255
SD	2.1898	0.4995	0.7191	1.2504	1.4062	2.0451

T_0 = control/ cake without DP; T_1 = 10% DP + 90% added sugar; T_2 = 20% DP + 80% added sugar; T_3 = 30% DP + 70% added sugar; SE = Standard Error; LSD = Least Significant Difference; SD = Standard Deviation; Mean values with different letters across rows are significantly different at ($p < 0.05$).

Table 4. Nutritional attributes of cake samples with different levels of sugar and date powder.

Treatments	Fiber %	Reducing sugar %	Non-reducing sugar %	Total sugar %	Energy value (kcal/100 g)
T_0	1.91 d	23.45 a	3.33 d	26.78 a	427.88 d
T_1	2.27 c	21.11 b	4.52 c	25.63 ab	452.27 c
T_2	2.97 b	19.03 c	5.12 b	24.15 b	468.00 b
T_3	3.51 a	17.23 c	6.85 a	24.08 b	478.47 a
SE ±	0.0216	0.8084	0.0242	0.8085	1.0724
LSD (0.05)	0.0498	1.8642	0.0557	1.8643	2.4730
SD	0.0498	2.4593	1.2698	1.3826	19.0816

T_0 = control/ cake without DP; T_1 = 10% DP + 90% added sugar; T_2 = 20% DP + 80% added sugar; T_3 = 30% DP + 70% added sugar; SE = Standard Error; LSD = Least Significant Difference; SD = Standard Deviation; Mean values with different letters across rows are significantly different at ($p < 0.05$).

ash content (5.77%), followed by T_2 (4.56%), T_1 (3.89%) and T_0 (1.89%). Carbohydrate content in cake samples ranged from 56.18% to 61.36%, with T_3 having the highest carbohydrate content (61.36%), followed by T_2 (59.98%), T_1 (58.49%) and T_0 (56.18%). Fiber content ranged from 1.91% to 3.51%, with T_3 showing the highest fiber content (3.51%), followed by T_2 (2.97%), T_1 (2.27%) and T_0 (1.91%). Reducing sugar content varied from 17.23% to 23.45%, with T_0 showing the highest reducing sugar content (23.45%), followed by T_1 (21.11%), T_2 (19.03%) and T_3 (17.23%). Non-reducing sugar content ranged from 3.33% to 6.85%, with T_3 having the highest non-reducing sugar content (6.85%), followed by T_2 (5.12%), T_1 (4.52%) and T_0 (3.33%). Total sugar content varied from 24.08% to 26.78% with T_0 showing the highest total sugar content (26.78%), followed by T_1 (25.63%), T_2 (24.15%), and T_3 (24.08%). The energy value ranged between 427.88 to 478.47 kcal/100g, with T_3 having the highest energy value (6.94 kcal/100g), followed by T_2 (468 kcal/100g), T_1 (452.27 kcal/100g) and T_0 (5.67 kcal/100g).

3.3. Sensorial Attributes of Cake Samples with Different Levels of Table Sugar and Date Fruit Powder

The results regarding average sensorial attributes of cake samples prepared with different levels of sugar and date powders are presented in Figure 1. The observed results revealed that all treatments showed statistically different ($p < 0.05$) mean values for sensory attributes. The color scores across treatments ranged from 6.33 to 8.33, with T_3 significantly outperforming other treatments at

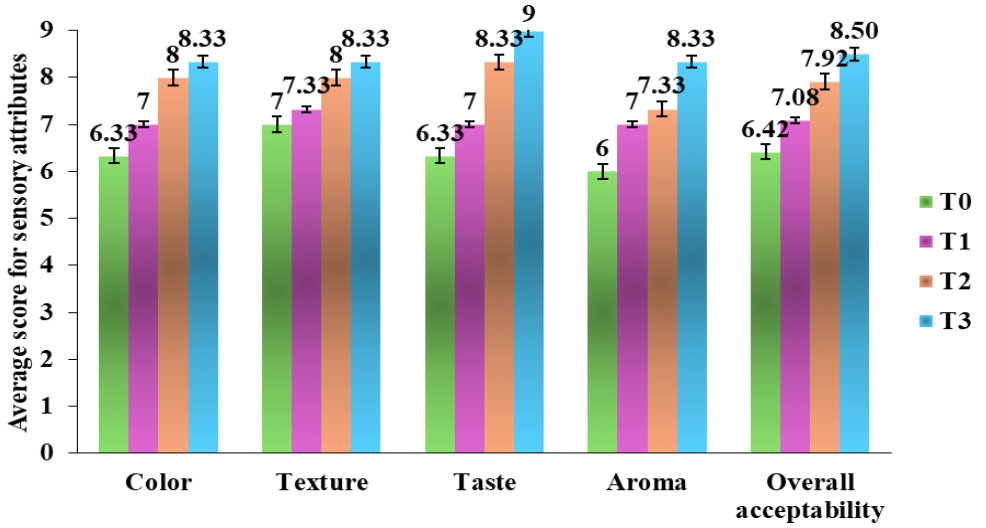

Fig. 1. Cake samples with different levels of table sugar and date powder.

8.33, followed by T_2 (8) and T_1 (7), while T_0 had the lowest score at 6.33. Scores for texture varied between 7 and 8.33, with T_3 having the highest score (8.33), followed by T_2 (8) and T_1 (7.33), while T_0 scored significantly lower at 7. For taste, scores ranged from 6.33 to 9, with T_3 leading at 9, followed by T_2 (8.33) and T_1 (7), while T_0 had the lowest score at 6.33. Scores for aroma ranged from 6 to 8.33, with T_3 scoring significantly higher at 8.33, followed by T_2 (7.33) and T_1 (7), while T_0 had the lowest score at 6. The scores for overall acceptability ranged from 6.42 to 8.50, with T_3 having significantly higher acceptability at 8.50, followed by T_2 (7.92) and T_1 (7.08), while T_0 had the lowest score at 6.42.

4. DISCUSSION

DP not merely supplies energy, promotes digestive health due to its immense fiber content, exerts antioxidant activity [20] but also adds pleasant sweetness in foods therefore can be effectively used as a healthier replacer of TS. DP offers huge health benefits instead to refined TS due to its nutrient richness, low glycemic index, etc., [21]. It was noticeable that DP of var. *Dhaki* showed to have a substantial proportion of energy, carbohydrate, and total sugars. Moreover, reducing sugars were extremely higher than non-reducing sugars. The chief carbohydrate in DP is sugar, i.e., monosaccharides (mainly glucose and fructose) as reducing sugars and disaccharides (sucrose) as non-reducing sugar [22]. The fiber content in DP found higher due to moisture removal during drying. In our findings, *Dhaki* DP contained substantial protein, which may be considered a reasonable quantity. Date fruit drying removes moisture resulting in a higher concentration of other components such as fiber, ash, protein, fats, etc., [23]. However, fat is one limiting component in date fruits [24]. The pH of DP was slightly acidic due to having different organic acids, i.e., malic, citric, and oxalic acid [5].

Cake is a popular food loaded with refined TS that links it to several health-related issues [25]. Present findings suggest that cake samples performed differently for nutritional characteristics and sensorial attributes due to having varying proportions of DP and TS. Among all cake samples, T_0 exhibited lower average values for fat, protein, ash, carbohydrate, and fiber content while energy value was also minimal, whereas moisture content and total sugars were found higher in T_0. The cake formulations in which TS was replaced with DP showed to have adequate levels of essential components such as fat, protein, ash, carbohydrate, and fiber content whereas the energy value was also higher. Among DP-added cake samples, T_3 performed appropriately for both nutritional and sensorial attributes. Fat serves as a shortening agent in cakes that elevates taste and calorific value. T_3 showed a higher percentage of fat indicating greater energy value than the counterpart samples. Studies by Mrabet *et al.* [26] show higher fat in cakes with date fruit. However, DP exhibited a low-fat content suggesting it is a poor source of fat. Majzoobi *et al.* [27] observed higher ash in date fruit press cake. Mrabet *et al.* [26] reported ash content in date-

enriched muffins ranging from 0.96 to 1.07 g/100g. However, in the current study higher ash was noted in T_3 and DP. Dried date fruit contains fiber ranging from 8.09 to 20.25 g/100g [28], therefore, fiber remained higher in T_3. In this study, the cake samples were subjected to sensorial evaluation and the result showed that T_3 perceived a maximum average score for color, texture, taste, aroma, and overall acceptability. This proves that replacing TS with DP imparted richness in the sensorial attributes of the cake samples. It can be therefore interpreted that replacing TS with DP enhanced the overall nutritional and sensorial attributes of cake samples. DP can be a promising substitute for refined TS in cakes since it is concentrated with essential health-promoting components [23].

5. CONCLUSIONS

It is concluded from the present study that all cake samples attributed suitable nutritional properties and sensorial attributes. Among all treatments, T_3 (with the highest DP content, i.e., 30%) exhibited superior outcomes for most nutritional attributes followed by T_2 and T_1. Moreover, T_3 showed to have significantly higher average values for all sensorial attributes followed by T_1 and T_2. These findings suggest that DP can be a vital TS replacer in various foods for potentially enhancing their nutritional value without compromising sensory enjoyment.

6. RECOMMENDATIONS

Keeping in mind, the facts obtained from the present study that cake samples replaced with date powder against added sugar are nutritious and palatable therefore these can be commercialized to a market level so that consumers may get access.

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

8. ETHICAL STATEMENT

The present study did not involve any ethical issues concerning to human or animal subjects. All materials utilized were plants-sourced and no harmful procedures were performed. The study focused on the formulation and assessment of a food product (cake) with a natural sugar substitute (date fruit powder).

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Research Article

Preliminary Phytochemical, Antioxidant and Antimicrobial Investigation of Selected Medicinal Plants of Khyber Pakhtunkhwa

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Abstract: The majority of the people living in rural areas are still relying on traditional medicine for their primary healthcare needs. The locally available medicinal plants contain bioactive compounds that are widely used for the treatment of various chronic diseases caused by multidrug-resistant (MDR) microbes. However, research in this area has been limited. In this regard, the current study was designed on assessing the antimicrobial properties and phytochemical constituents of ethanol, methanol, hexane, ethyl acetate, and aqueous extracts of *Ruellia tuberosa, Aesculus indica,* and *Myrsine africana* against MDR microbes. The results of phytochemical analysis revealed the presence of different classes of secondary metabolites such as alkaloids, flavonoids, saponins, tannins, steroids, terpenoids, and reducing sugar, though anthraquinons, phlobatanins, and glycosides were absent in the selected plants. Furthermore, the quantitative analysis of methanol extract showed that *M. africana* had notable concentrations of total phenol (3.75 ± 0.05 mg $100g^{-1}$) and total flavonoid (11.39 ± 1.72 mg $100g^{-1}$) contents, while *R. tuberosa* had the highest (0.74 ± 0.06 mg $100g^{-1}$) tannins content. In addition, the lowest concentrations of total phenol (2.88 ± 0.04 mg $100g^{-1}$) and flavonoid (6.77 ± 1.02 mg $100g^{-1}$) were examined in *A. indica*, while the lowest tannin (0.31 ± 0.03 mg 100^{-1}) moiety was observed in *M. africana*. Moreover, the mean highest (46.64%) antioxidant activity was observed for *A. indica*, while the lowest value was observed for *M. africana* (32.56%). The antimicrobial susceptibility test showed that ethanol, hexane, ethyl acetate, and aqueous extracts of the selected plants had remarkable inhibitory potential against the test bacterial and fungal species. The antimicrobial activity of selected plant extracts had no to low and moderate to good inhibitory potential against the test microorganisms.

Keywords: Phytochemical, Total Phenol, Total Flavonoid, Antioxidant, Antimicrobial.

1. INTRODUCTION

Plants serve as a major source of therapeutic compounds that play a crucial role in traditional medicine recipes in remote regions of Pakistan. They provide essential elements for diets, medicines, pharmaceuticals, and food additives, and serve as the basis for various synthetic drugs [1]. Approximately 80% of the global population relies on natural resources for their primary healthcare needs, according to the World Health Organization (WHO). These plants, which have a repertoire of diverse bioactive compounds, are often considered weeds in Pakistan. Similarly, rural populations in India, Iran, Afghanistan, China, and other parts of Asia heavily depend on wild plants for their medicinal needs [2, 3]. Recent research highlights that many commonly used drugs are derived from plants or other natural sources [4]. Plant-derived secondary metabolites are extensively used not only for the treatment of various infectious diseases but also for curing cancer, allergy, inflammation, stress, diabetes, atherogensis, and thrombosis, as well as scavenging free radicals from the body [5, 6]. These pharmacological activities were attributed to the presence of various classes of secondary metabolites that are widely found in different parts of the plants, such as leaves, stems, roots, bark, flowers, fruits, and rhizomes [7]. Phytochemicals, including terpenoids, flavonoids, and carotenoids, contribute to these biological activities [8]. For example, flavonoids, a prominent class of polyphenols extensively found in plants, exhibit a multitude of pharmacological effects [9]. Within

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flavonoids, different subclasses such as flavones, flavonols, flavanones, isoflavones, isoflavans, coumestans, anthocyanins, and pterocarpans exist [10]. Numerous research studies have demonstrated the efficacy of these phytochemicals against various human, livestock, and poultry pathogens within traditional medicinal practices. However, both common and emerging infectious diseases pose significant threats to communities, highlighting the urgent need to identify diverse chemical compounds with improved mechanisms of action against these infections [11].

The free radicals such as hydroxyl, nitric oxide, hydrogen peroxide, and various singlet oxygen molecules are produced by our body during metabolic activities [12, 13]. These free radicals are the major cause of many chronic and degenerative diseases [14]. The oxidative damage caused by free radicals is prevented by antioxidants. The antioxidant compounds are predominantly produced by plants in the form of secondary metabolites such as phenols, vitamins, terpenoids, and flavonoids [15]. Many secondary metabolites produced by plants can also serve as effective drugs against harmful microbes and unwanted cells [16]. Recently, there has been a focus on screening antimicrobial activities due to the rise in antibiotic-resistant infectious agents, leading to therapeutic challenges. Plant-derived antimicrobial compounds have proven highly potent even in small doses, often outperforming synthetic alternatives under similar evaluation methods [17, 18]. The presence of secondary metabolites in plants has long been recognized, serving as inspiration for the development of innovative drug compounds and making significant contributions to human health through natural products derived from plants. Therefore, this study was designed to screen the organic extracts of selected plants for the existence of different classes of secondary metabolites that contribute to antimicrobial and antioxidant activities.

2. MATERIALS AND METHODS

2.1. Plants Collection

In this study, the roots of *R. tuberosa,* nuts of *A. indica*, and aerial parts of *M. africana* were collected from Charsadda, Peshawar, and Swat districts of Khyber Pakhtunkhwa province, Pakistan. The respective plant parts were thoroughly washed with tap water, followed by distilled water, to remove all kinds of adhering materials. The selected part of each plant was stored under shade for complete dryness. The dried plant materials were converted to powder form by using an electrical grinder (Yigan, model WF130). The resulting powdered samples were stored at 4 °C after carefully packing and labeling them in clean Zip-lock bags [19]. The phytochemical and antimicrobial activity was performed at the Department of Agricultural Chemistry, the University of Agriculture Peshawar, and the Pakistan Council of Scientific and Industrial Research Institute (PCSIR), Peshawar, Pakistan.

2.2. Extraction and Fractionation

The extraction was performed by adding ethanol (2.0 l) to the powdered sample (1.50 kg) taken in the separating funnel, employing the cold maceration technique. The crude ethanol extract was collected from the separating funnel and filtered through Whatman filter paper No. 1. The filtrate was then dried at 45 °C using a rotary vacuum evaporator (Heidolph Laborota 4000). Afterward, 100–150 ml of distilled water was added to the dried ethanol extract and then partitioned with hexane and ethyl acetate. The dried hexane and ethyl acetate fractions were obtained by evaporating the respective solvents through a rotary evaporator. The dried ethanol extract and its fractions, i.e., hexane, ethyl acetate, and aqueous, were kept in clean glass vials and stored in the refrigerator at 4 °C for further analysis [20].

2.3. Phytochemical, Antioxidant and Antimicrobial Analysis

The crude ethanol extract and fractions of the selected plants were screened for the presence or absence of different classes of secondary metabolites by the method of Trease and Evans (1989) [21]. Similarly, total phenol and tannin contents were quantitatively estimated using the method of Grubesic *et al.* [22]. Likewise, the total flavonoid content was determined by the method described by Sharma *et al.* [23], while the standard protocol of Akond *et al.* [24] was used for the determination of antioxidant activity. The antimicrobial potential of the extracts was tested against six gram-negative bacteria (i.e., *Klebsiella pneumonia* (ATCC # 13883), *Pseudomonas aeruginosa* (ACCT # 9721), *Escherichia coli* (ACCT # 25922), *Erwinia*

carotovora, Salmonella typhi, and *Agrobacterium tumefacien*), three gram-positive bacteria (*Bacillus subtilis* (ATCC # 6633), *Staphylococcus aureus* (ATCC # 6538), and *Bacillus atropheous*), and one fungal strain, *Candida albicans,* by the disc diffusion method of Bauer *et al.* [25]. The inhibitory zone of the plant extracts was recorded in millimeters (mm) and then expressed in percentages by using the formula: inhibition (%) = sample/control × 100.

2.4. Statistical Analysis

The experiment was laid out in a 2-factorial completely randomized design (CRD) using Statistix 8.1 statistical software. All the data were recorded in triplicate. Mean comparisons were conducted using the least significant difference (LSD) test at a significant level of 0.05.

3. RESULTS AND DISCUSSION

3.1. Phytochemical Screening

The data of phytochemical analysis revealed that crude ethanol, hexane, ethyl acetate, and aqueous fractions of *R. tuberosa, A. indica,* and *M. africana* contain different classes of secondary metabolites such as flavonoids, terpenoids, tannins, and steroids, respectively. Likewise, phlobatnin, glycoside, and anthraquinon were not found in the said plant extracts. In addition, alkaloids were found in the hexane, ethyl acetate, and aqueous extracts of *R. tuberosa* but did not exist in any of the extracts of *A. indica* and *M. africana*. Saponin and reducing sugar were present in all plant extracts except hexane, ethyl acetate, and aqueous fractions of *R. tuberosa* (Table 1). The results of phytochemical screening observed in the course of this study are in close proximity with the earlier reported studies [26-28]. The rationale behind this study lies in the role of initial understanding of phytochemicals in standard herbal drug development processes. This preliminary knowledge of phytochemicals provides insights into the diverse chemical constituents found in plant materials [29]. Moreover, exploring the presence of bioactive agents in plants not only informs but also provides potential precursors for the development of synthetic drugs [30]. The results of total phenol, tannin, and flavonoid content of the selected plants are presented in Table 2. The data showed that *M. africana* had a remarkable concentration of total phenol (3.75 ± 0.05 mg $100g^{-1}$) and total flavonoid (11.39 ± 1.72 mg $100g^{-1}$) contents, while *R. tuberosa* had the highest (0.74 ± 0.06 mg $100g^{-1}$) tannin content. Moreover, the lowest concentrations of total phenol (2.88 ± 0.04 mg $100g^{-1}$) and flavonoid (6.77 ± 1.02 mg $100g^{-1}$) were examined in *A. indica,* while the lowest tannin (0.31 ± 0.03 mg $100g^{-1}$) content was observed in *M. africana*, respectively. ANOVA showed that the values of total phenol, tannin, and total flavonoid contents varied significantly at $p < 0.05$. The results obtained in the course of this study find supportive evidence from previous findings [26, 27, 31-33].

3.2. Antioxidant Activity

The results of the antioxidant activity of selected

Table 1. Bioactive constituents in different extracts of *R. tuberosa, A. indica,* and *M. africana*.

S. no.	Bioactive constituents	Ethanol			Hexane			Ethyl acetate			Aqueous		
		RT	AI	MA	RT	AI	MA	RT	AI	MA	RT	AI	MA
1	Alkaloid	-	-	-	+	-	-	+	-	-	+	-	-
2	Tannin	+	+	+	+	+	+	+	+	+	+	+	+
3	Flavonoid	+	+	+	+	+	+	+	+	+	+	+	+
4	Phlobatnin	-	-	-	-	-	-	-	-	-	-	-	-
5	Steroid	+	+	+	+	+	+	+	+	+	+	+	+
6	Terpenoid	+	+	+	+	+	+	+	+	+	+	+	+
7	Saponin	+	+	+	-	+	+	-	+	+	+	+	+
8	Reducing sugar	+	+	+	+	+	+	-	+	+	-	+	+
9	Glycoside	-	-	-	-	-	-	-	-	-	-	-	-
10	Anthraquinon	-	-	-	-	-	-	-	-	-	-	-	-

RT = *Ruellia tuberosa*, AI = *Aesculus indica*, MA = *Myrsine africana*.

Table 2. Total phenols, tannins, and flavonoid contents of methanol extracts of *R. tuberosa, A. indica,* and *M. africana*.

Plants	Total phenol content (mg $100g^{-1}$)	Total tannin content (mg $100g^{-1}$)	Total flavonoid content (mg $100g^{-1}$)
R. tuberosa	3.41±0.07b	0.74±0.06a	10.43±0.98b
A. indica	2.88±0.04c	0.53±0.05b	6.77±1.02c
M. africana	3.75±0.05a	0.31±0.03c	11.39±1.72a

Values in each column are the mean of three replication ± standard deviation. Mean in the column followed by different alphabets are significant at $p < 0.05$.

plants at different concentrations are described in Figure 1. The antioxidant potential of *R. tuberosa, A. indica*, and *M. africana* ranged from 10.78 to 77.41%, 11.78-80.41%, and 6.41 to 74.36%, respectively. It was observed that antioxidant potential increased with increasing concentrations of plant extracts. The %DPPH values of selected plants varied significantly at $p < 0.05$. The antioxidant potential of *R. tuberosa, A. indica,* and *M. africana* observed in this study is in close proximity to the previous studies [34-35]. A plethora of studies have revealed that the presence of free radicals in the body can lead to various chronic and degenerative diseases like diabetes, cancer, coronary heart disease, inflammation, stroke, etc. [36]. Antioxidants play a crucial role in preventing oxidative damage caused by free radicals. They inhibit the oxidation process by interacting with free radicals, catalytic metals, and chelating agents, as well as by acting as oxygen scavengers [37]. The DPPH is a rapid, consistent, and reproducible method extensively used for the search for antioxidants not only in plant extracts but also in pure compounds [38].

3.3. Antimicrobial Activity

The antimicrobial activities of different extracts of *R. tuberosa* (Figure 2**)** showed that ethyl acetate had the highest inhibitory activity against *B. atropheous* (69.36%) and *E. carotovora* (68.64%), followed by aqueous extract against *B. subtilis* (68.43%), ethanol extract against *B. atropheous* (68.35%), and hexane extract against *E. carotovora* (65.81%). Furthermore, ethyl acetate and aqueous extracts had no inhibitory potential against *K. pneumonia* and *E. carotovora*. The ANOVA showed that *R. tuberosa* extracts significantly ($p < 0.05$) inhibited the growth of selected microbial strains. The results obtained in this research work were in accordance with previous research that described the notable inhibitory potential of alcohol, chloroform, ethyl acetate, and water extracts against different opportunistic microbial strains [39]. In a similar study [40], promising inhibition of the methanol extract of the said plant was observed against *E. coli, P. aeruginosa, K. pneumonia,* and *B. subtilis,* which is supportive evidence of our findings.

The results of the percent inhibition potential of *A. indica* (Figure 3) revealed that ethyl acetate extract had a notable inhibitory zone against *S. aureus* (69.40%), *E. carotovora* (60.70%), and *P. aeruginosa* (50.89%), whereas ethanol, hexane, and aqueous extracts had >50% inhibitory activity

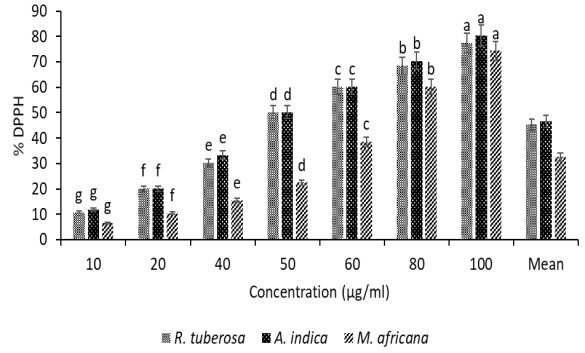

Fig. 1. Antioxidant activity of different concentrations of methanol extracts of *R. tuberosa, A. indica,* and *M. africana*.

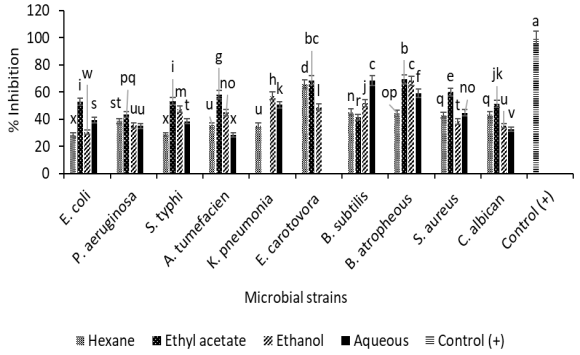

Fig. 2. Inhibitory potential of crude ethanol extract and fractions of *R. tuberosa* against pathogenic microbial strains.

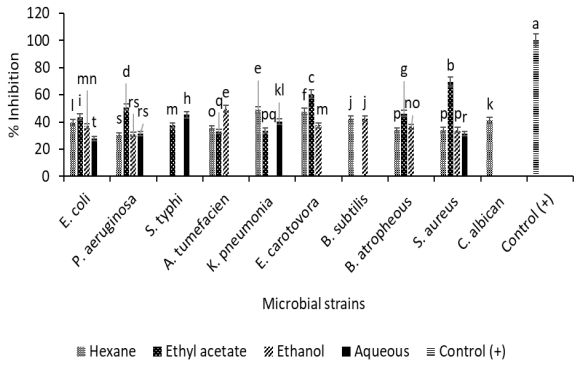

Fig. 3. Inhibitory potential of crude ethanol extract and fractions of *A. indica* against pathogenic microbial strains.

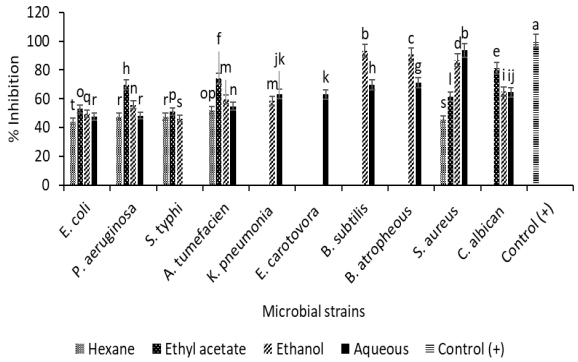

Fig. 4. Inhibitory potential of crude ethanol extract and fractions of *M. africana* against pathogenic microbial strains.

against the test microbial strains. It was observed that some microbial species showed resistance to the said plant extract and displayed no inhibition activity. The ANOVA showed that *A. indica* extracts significantly ($p < 0.05$) inhibited the growth of selected microbial strains. The results of our study are on par with earlier findings, which showed promising inhibitory zones of methanol and aqueous extracts of the said plant against *S. aureus* and *B. subtilis* [41]. Similarly, the highest inhibitory zones of 20.0 mm and 18.0 mm for ethanol and aqueous extracts of *A. hippocastenum* were noted against *S. mutans* and *S. sanguis* [42].

Likewise, the antimicrobial ability of *M. africana* (Figures 4) revealed that the aqueous extract had the highest inhibition potential against *S. aureus* (93.56%), followed by the ethanol extract, which showed a notable percent inhibition against *B. subtilis* (93.24%), and *B. atropheous* (90.49%). The said plant extracts showed good to moderate and low to no inhibitory activities against the test microbial strains. The ANOVA showed that *M. africana* extracts significantly ($p < 0.05$) inhibited the growth of selected microbial strains. Earlier studies showed that methanol, hexane, and chloroform extracts of *M. africana* had good to moderate and low to no activity against the test pathogenic bacterial strains [43]. In another research work, the hexane, chloroform, and ethanol extracts of the leaves, stems, and roots of *M. africana* were screened for an antimicrobial susceptibility test. The results showed that all extracts except hexane obtained from stem and root exhibited considerable inhibitory activity against the test microbial strains. In addition, chloroform extract has shown more antimicrobial activity than ethanol extract [44].

Moreover, the standard drugs, i.e., levofloxacin, ciprofloxacin, and clotrimazole, used as positive controls against gram-negative, gram-positive, and fungi, exhibited 100% inhibitory potential against the test microbial strains. Likewise, pure dimethyl sulfoxide used as a negative control did not inhibit the growth of selected pathogenic microbial strains (Figures 5).

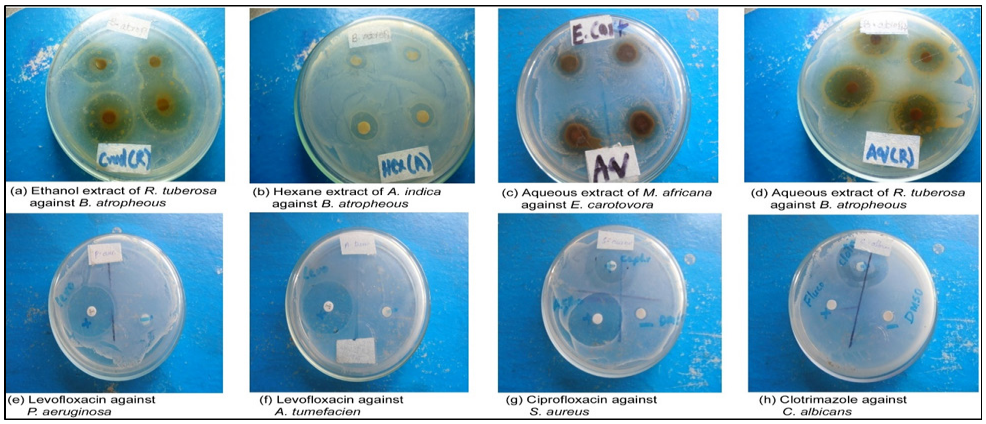

Fig. 5. Images of selected plant extracts and standard antibiotic against different microbial strains.

4. CONCLUSIONS

The results of the phytochemical analysis indicate that the selected plants are effective sources of various classes of secondary metabolites such as total phenols, total tannins, total flavonoids, etc. The selected plants exhibited notable antioxidant and antimicrobial activities, suggesting their potential as sources of antibiotics for treating various illnesses and for utilization in the pharmaceutical and cosmetic sectors. This study underscores the importance of isolating and identifying novel secondary metabolites to assess their effectiveness against different diseases.

5. CONFLICT OF INTEREST

The author has no conflict of interest.

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Pakistan Academy of Sciences

Research Article

Microbe Mediated Extraction of Keratin from *Gallus gallus domesticus* and its Role as a Fertilizer and in Poultry Feed

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Abstract: The poultry industry in Pakistan generates substantial feather waste, contributing to environmental contamination. This study aimed to isolate keratinolytic bacteria from chicken (*Gallus gallus domesticus*) feathers and soil samples. Twelve isolates exhibited proteolytic activity, forming clear zones on skim milk agar and growing efficiently on minimal agar, suggesting they belong to the Bacillus species. One promising strain, identified through 16S rRNA sequencing as *Pseudomonas aeruginosa*, demonstrated optimal feather degradation at pH 7.0-9.0 and temperatures of 37 °C and 45 °C. Protein content was measured as 4.8 mg/ml for chicken feathers and 5.43 mg/ml for quail feathers ($p < 0.05$). The FTIR spectrum of keratin hydrolysate for the chicken feathers was recorded within 4000-7000 cm^{-1}. Strain SC7 showed significant plant microbial interaction with tomato (*Solanum lycopersicum*), pea (*Pisum sativum*), and cucumber (*Cucumis sativus*), with dilutions of 100x (0.048 mg/ml) and 70x (0.068 mg/ml) promoting the growth of tomato, pea, and cucumber. When used as a feed additive, keratin significantly ($p < 0.05$) increased chicken body weight by 67.7% compared to controls. Liver function tests (ALT, AST, ALP, total protein, albumin, globulin, albumin/globulin ratio) showed decreased ALT and AST and increased ALP, total protein, albumin, and globulin in the experimental group compared to controls. Histopathology revealed no degenerative effects of keratin on chicken liver hepatocytes.

Keywords: *Bacillus, Pseudomonas aeruginosa,* Hydrolysate, Keratin Feed, FTIR, LFT, Histopathology, Chicken.

1. INTRODUCTION

Poultry industry is growing worldwide, and large amounts of excreta are coming from this industry [1]. With an average yearly rise of 1.7% since 2017, Pakistan's poultry meat production is expected to increase from 1.45 million metric tons in 2021 to 1.6 million metric tons by 2026 [2]. However, disposal of feathers is a major concern for the poultry industry and accumulation of this huge volume of the feathers results in environmental pollution and protein wastage [3]. It is well known that feathers are a very rich source of protein with β-keratin constituting 91 % of feather protein *Gallus gallus domesticus* [4]. Keratin is an insoluble protein molecule having very high stability and low degradation rate [5]. Based on secondary structural confirmation, keratins have been classified into α keratin (α-helix in hair and wool) and ß keratin (ß-sheets in feather) [6]. Many microbes like *Bacillus* spp., *Burkholdreia, Chryseobacterium, Pseudomonos, Microbacterium* spp. can degrade keratin by producing keratinase [7, 8]. Essential amino acids can be obtained from degraded feathers and act as precursor for the compounds which promote plant growth [9]. Replacing hazardous synthetic fertilizers with eco-friendly bio-fertilizers can be an alternative option for enhancing agricultural productivity [10]. A minor quantity of poultry feathers started to be used in other industrial applications such as clothing, insulation and bedding [11]. Presently, keratin hydrolysates are also in use in formulation of feed for livestock and fertilizer for agriculture industry [12]. There is scarce data available regarding isolation of bacteria, feather degradation, keratin estimation and its application as a fertilizer and feed in plants and animals respectively. The present study was conducted to isolate and characterize keratinolytic bacteria from the soil samples collected from dumpsite of a private poultry shed. This study is useful for converting poultry waste into value

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added products in the environment. The keratin hydrolysate was used as fertilizer for different plant species such as tomato (*Solanum lycopersicum*), Pea (*Pisum sativum*), and Cucumber (*Cucumis sativus*). Moreover, the keratin hydrolysate was also used as a feed additive for chicken.

2. MATERIALS AND METHODS

2.1. Collection of Samples and Preparation of Feather Powder from *Gallus gallus domesticus*

Feathers of *Gallus gallus domesticus* (Chicken) were collected from a local chicken shop along with dry soil from dumpsite of a private poultry shed in Jahman village near Lahore. The samples were collected in the month of May 2021 in a zipper plastic bag. After collecting feather samples these were autoclaved for 15 minutes at 121 ºC and dried in hot air oven for 4 hours at 50 ºC. The dried feathers were crushed in the grinder machine to obtain fine powder. The yield of keratin powder obtained from feathers was calculated by formula:

$$Yield = \frac{amount\ of\ feathers\ powder}{amount\ of\ feathers} \times 100$$

2.2. Isolation, Purification and Screening of Bacterial Isolates from Poultry Soil

Bacterial isolation from poultry feathers and soil was done by culture enrichment technique according to Kumar *et al.* [6]. Almost 30 different colonies were obtained and purified by quadrant streak. The purified colonies were further tested for their proteolytic activity and isolates were cultured on Skim milk Agar [13]. The plates were incubated for a week at 37 ºC and isolated based on clear zones. Almost twelve isolates were obtained and cultured on minimal growth media and named as SC1, SC2, SC3, SC4, SC5, SC6, SC7, SC8, SC9, SC10, SC11 and SC12.

2.3. Characterization and 16S rRNA Sequencing Bacterial Isolates

Selected bacterial isolates were characterized both morphologically and biochemically according to methods of Cappuccino and Sherman [14]. From all twelve isolates the genetic identification of only one isolate SC7 was carried out using 16S ribosomal gene sequence analysis using commercial services of IBM.

2.4. Preparation of Hydrolysate

Protein hydrolysate was prepared with the help of isolated microbes and biodegradation as carried out under controlled conditions at specific pH, and temperature. Later, this hydrolysate was used in the experiment as a protein source.

2.5. Effect of Temperatures and pH on Degradation of Feathers

All isolates were cultured at varying temperatures such as 25 ºC, 37 ºC and 45 ºC and pH 5.0, 7.0 and 9.0. The effect of isolates on the possibility of degradation was recorded for chicken and quail feathers. For this the isolated bacteria were incubated in LB-Broth at 37 °C for 24 hours. After incubation, Optical Density was taken at 600 nm and different parameters such as stereomicroscopy, protein estimation, FTIR, biofilm assay was monitored.

2.5.1. Stereomicroscopy

Samples of hydrolysate (1 ml) were analyzed using stereomicroscope before and after experiment completion. A stereomicroscope is a low-magnification optical instrument that uses two distinct optical channels to provide a three-dimensional picture of specimens. With improved depth perception, it is frequently employed to examine the surfaces of materials such as hydrolysate in this study.

2.5.2. Protein estimation

To study the Protein contents of degraded feathers (keratin obtained) by bacterial isolates, were determined using Bovine Serum Albumin (BSA) as the standard Lowry *et al*. [15]. The optical density (OD) was recorded at 750 nm. Optical Density is the amount of light absorbed by a sample as it passes through a medium. It is commonly used to measure the concentration of a solution and indicates the attenuation of light.

2.5.3. Biofilm assay

Biofilm formation of selected isolates was done

according to the methods of Qurashi and Sabri [16]. After 4 days, OD at 600 nm was observed for planktonic cells. Optical density of biofilm in term of adhered cells was recorded at 570 nm.

2.5.4. Fourier Transform Infrared (FTIR) spectroscopy analysis

The FTIR spectrum of the extracted keratin hydrolysate was analyzed for the detection of amide groups and functional groups. The Fourier Transform Infrared (FTIR) Spectrophotometer measures the infrared absorption of materials to identify and classify them. It operates on the premise that certain infrared light frequencies are absorbed by molecules, which causes their bonds to vibrate. Each substance's molecular fingerprint is provided by these absorption patterns. Keratin hydrolysates were sent to a private Laboratory for getting commercial services in Lahore, Pakistan and the result were obtained and noted.

2.6. Assessment of Plant Microbe Interaction (PMI)

The bacterial isolates (SC1, SC2, SC3, SC4, SC5, SC6, SC7, SC8, SC9, SC10, SC11 and SC12) were inoculated in autoclaved sterilized LB-Both and then incubated at 37 ºC for 24 hours. The incubated LB-Broth was then centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded, and pellet was suspended in the normal saline. The seeds of tomato (*Solanum lycopersicum*), and Cucumber (*Cucumis sativus*) were sterilized with 0.1% $HgCl_2$ and soaked in pellet suspended in normal saline for 20 minutes. These seeds were placed in sterilized petri dishes, which have wet filter paper. The germination setup had 5 seeds of each plant/ isolate. These plates were kept in dark for 3 days until the germination. The dry weight and fresh weight of seedling was measured along with root and shoot length. Then plants were allowed to grow in sunlight. The growth was observed for the next 14 days, after that root and shoot length was again measured. Control seeds were not treated with pellets.

2.6.1. Use of keratin as a fertilizer

Keratin hydrolysate was used as a fertilizer and methods of Kshetri *et al*. [17] were followed with slight modifications. Keratin hydrolysate was filtered using a sieve of 0.2 mm to remove the undigested feathers. The seedlings were placed in dilutions of keratin hydrolysate for aqua culture and 5 plants per dilution were allocated to each set up. The certified seeds of tomato (*Solanum lycopersicum*), Pea (*Pisum sativum*), Cucumber (*Cucumis sativus*) were collected from Punjab Seed corporation Lahore Pakistan. The seeds were sterilized with 0.1% $HgCl_2$ and placed in petri dishes, with wet filter papers. The germination setup had 5 seeds of each plant. These plates were kept in dark for 3 days until the germination. Then plants were allowed to grow in sunlight, the growth was observed for the next 14 days. The control setup with 5 plants was placed in distilled water. The number of germinated seeds, root lengths, and shoot lengths were recorded and compared with both experimental and control setup.

2.7. Keratin as Poultry Feeds

Keratin was also used as a poultry feed as described by Fisinin *et al*. [18]. Feed was prepared in 50% w/v ratio. The feed was soaked in keratin hydrolysate and then air dried. There were two groups, one control and another was experimental. Birds of 10 days were placed in triplicates. The control group was fed by the regular poultry feed while experimental set was fed with feed that was soaked in keratin hydrolysate. The initial and final body weight were recorded. The body weight gain was calculated for both sets and compared.

2.7.1. Liver function test

The purpose of liver function test was to determine the liver metabolism, injury of a cell or response to the injury. The liver function test of chicks of both control and experimental setup was done. For this the blood samples of birds from both groups were collected and send to a private laboratory in Lahore, Pakistan. The parameters such as BILI (bilirubin), ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), TP (total protein), ALB (albumin), GLOB (globulin) and A/G (albumin/globulin) were recorded.

2.7.2. Histopathology of chick liver

Histopathology of liver was performed and for this purpose birds were slaughtered, and liver samples were collected, washed, labelled, and preserved

in 10% formalin. The histopathology test of liver samples was performed to check the efficacy of keratin hydrolysate that was obtained from microbial degradation of chicken feathers. For The samples were sent to private Laboratory for getting commercial services in Lahore, Pakistan and the result were obtained and noted.

3. STATISTICAL ANALYSIS

Statistical analysis was performed using mean, standard error, and least significant difference (LSD) tests using MS Excel 2016. Statistical analysis was performed for each parameter using standard error to calculate the mean and standard deviation. Analysis of variance (ANOVA) was conducted to assess significant differences, and the least significant difference (LSD) was applied for post hoc comparisons. All analyses were carried out using SPSS version 22.

4. RESULTS

4.1. Preparation of Feather Powder from *Gallus gallus domesticus* and Isolation of Bacteria

Feathers of *Gallus gallus domesticus* (Chicken) were finally crushed and changed to fine powder. While bacterial isolates from enrichment cultures were purified by streaking and then selected isolates were screened on skim milk agar for proteolytic activity. From 30 different bacterial colonies, that showed positive results in the form of clear zones on skim milk agar were purified by streaking and twelve isolates SC1, SC2, SC3, SC4, SC5, SC6, SC7, SC8, SC9, SC10, SC11 and SC12 were selected and characterized for their morphological and biochemical characteristics. It was noticed that all the isolated strains belonged to bacillus genus.

4.2. 16S rRNA Sequencing

Genetic identification and phylogenetic relationship of one of active bacterial isolates was described by using sequence analysis of 16S rRNA gene. The 16S rRNA gene sequence of SC7 785F strain was submitted to NCBI genbank and accession number was obtained which was ACCESSION OM846627. Phylogenetic tree based on 16S rRNA gene partial sequence of bacterial strain SC7 using the Neighbor-Joining method [19] was obtained. The results of 16S r RNA gene sequence (1457 bp) of SC7 showed closest (100%) similarity to *Pseudomonas* strain DSM 5425 and it was identified as *Pseudomonas sp.* strain, as shown in Figure 1.

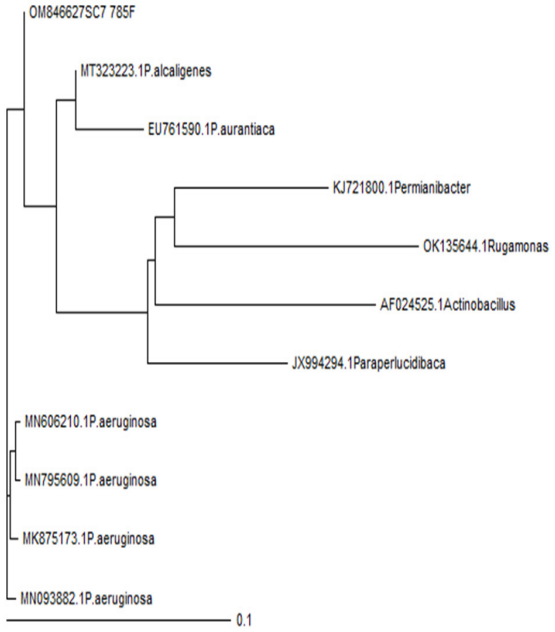

Fig. 1. Phylogenetic tree based on 16S rRNA gene partial sequence of bacterial strain SC7 using the Neighbor-Joining method [19].

4.3. Growth in Minimal Agar and Effect of Temperatures and pH on Bacterial Growth

The isolates were streaked on minimal agar plates and results were observed after 24 hours, all isolates showed noticeable growth in minimal media. Then the effect of varying pH conditions on bacterial growth was checked and results showed a general trend of significant ($p < 0.05$) increase in cell densities at pH 9.0 as compared to pH 5.0 and pH 7.0 with few exceptions. The analysis of cell densities showed that isolated SC7 showed the highest cell densities at pH 7.0 and pH 9.0 as compared to the results of other bacterial isolates and control treatment (Figure 2). Effect of varying temperature conditions on bacterial growth showed a significant ($p < 0.05$) increase in cell densities at temperature 37 ℃ and 45 ℃ as compared to temperature 25 ℃ for all bacterial isolates. The highest cell densities at different temperatures were shown by bacterial isolate SC8 that was significantly high ($p < 0.05$) as compared to other bacterial isolates and control treatment (Figure 3).

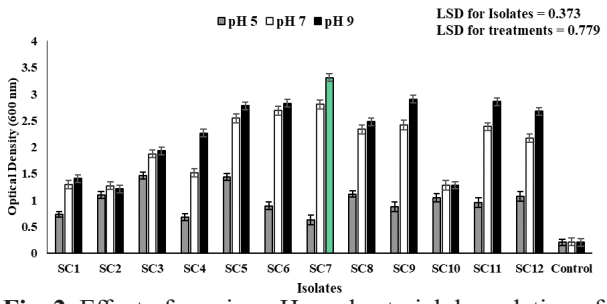

Fig. 2. Effect of varying pHs on bacterial degradation of feathers.

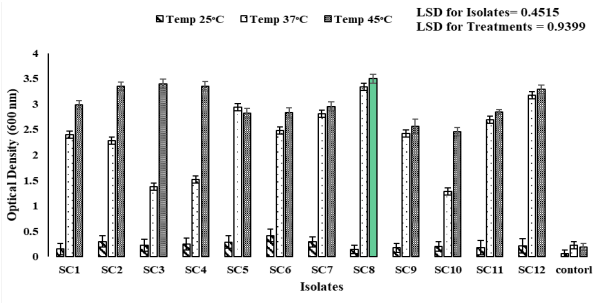

Fig. 3. Effect of Temperature on bacterial degradation of feathers.

4.4. Effect of Temperature and pH on Bacterial Degradation of Feathers

It has been observed that an increase in temperature resulted in increased feathers degradation with bacterial inoculation, and time of degradation was reduced as compared to control. An increase in temperature reduced the time for feathers degradation from 4 weeks to almost 3 weeks. Degradation of feathers was done in 3rd week at temperature 45 °C. While, at temperature 37 °C and 25 °C four and eight weeks were required for the feathers degradation in hydrolysate. Moreover, degradation of feathers completed at pH 5.0, 7.0 and 9.0 in 4th week in hydrolysate. Degradation of feathers substrate was found to be associated with significant increase ($p < 0.05$) in pH of the medium to alkalinity, thus serving as an indicator for the efficiency of degradation. Generally, an increased pH was observed toward completion of the experiment and media became alkaline despite adjusted pHs and temperatures.

4.5. Stereomicroscopy

The stereomicroscope was carried out at the start and end of the feathers' degradation, and it was noticed that degraded components were clearly visible in microscope as compared to control treatment.

4.6. Protein Estimation and Biofilm Assay

Bacterial Isolates obtained from *Gallus gallus domesticus* feathers and soil showed positive biofilm formation in 4 days at temperature 45 °C and pH 9.0. Optical density of biofilm in terms of adhered cells were recorded at 570 nm. The highest optical density was recorded by bacterial isolates SC7 and SC8 (Figure 4). The protein content of *Gallus gallus domesticus* feathers at 45 °C was 4.64 mg/ml and 4.8 mg/ml for bacterial isolates SC7 and SC8, respectively (Figure 5). At pH 9.0 protein content was 5.5 mg/ml and 5.43 mg/ml for bacterial isolates SC7 and SC8, respectively (Figure 6).

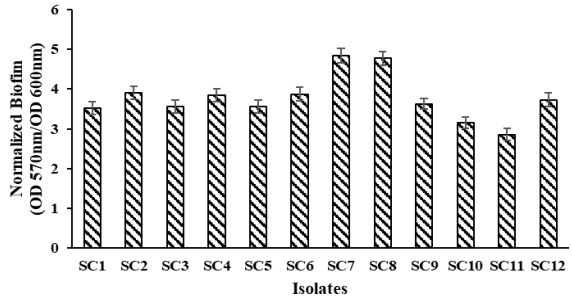

Fig. 4. Optimized biofilm bacterial isolates collected from Chicken feathers (*Gallus gallus domesticus*) at pH 9.0 and temperature 45 °C.

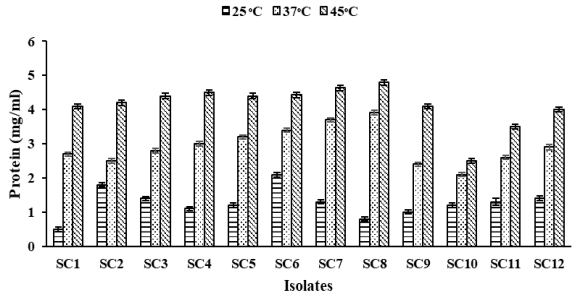

Fig. 5. Amount of protein content estimated from Chicken (*Gallus gallus domesticus*) feather at different temperatures.

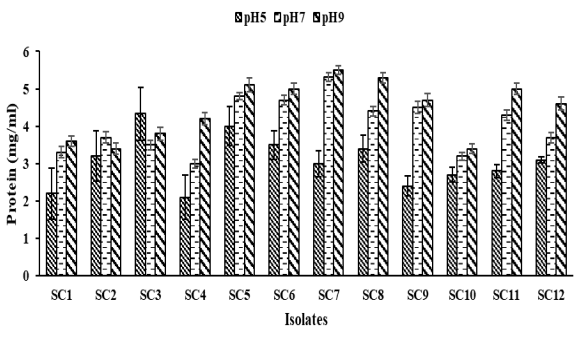

Fig. 6. Amount of protein content estimated from Chicken (*Gallus gallus domesticus*) on different pH.

4.7. Fourier Transform Infrared (FTIR) Spectroscopy Analysis

The FTIR spectrum was observed at 4000-700 cm^{-1} for both feathers. For keratin from chicken (*Gallus gallus domesticus),* the absorption peak range between 3500-3100 cm^{-1} (Figure 7) indicating O-H stretching. The absorption peak at range between 3000-2900 cm^{-1} showed C-H stretching represented amid-III group, peaks range between 1700-1600 cm^{-1} showed C=O stretching represented amid-I group, peaks range between 1450-1400 cm^{-1} showed N-H group represented amid-II group. Peaks at 877, 1085, 1043 cm^{-1} ranged between 1100-800 cm^{-1} showed S=O stretching represented cysteine.

4.8. Plant Microbe Interaction (PMI)

The plant microbe interaction experiments were carried out to check the efficacy of isolates in germination on tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus)* seeds. Plant microbe interaction showed that there was a general trend of significant ($p < 0.05$) increase in germination and growth of tomato and cucumber seeds as compared to control. The bacterial isolates SC7 and SC8 were highly significant ($p < 0.05$) for the tomato and cucumber seed germination and growth as compared to other bacterial isolates and control (Table 1). The bacterial isolates SC8 was significant ($p < 0.05$) for cucumber seeds in reference to fresh and dry weight and for tomato seed SC8 was significant for fresh weight and SC7 and SC8 were significant for dry weight as compared to other bacterial isolates and control (Table 2).

4.9. Keratin as Fertilizer

Keratin as fertilizer (at 50x, 70x and 100x) showed positive results for all 3 plants, tomato (*Solanum lycopersicum*), pea (*Pisum sativum*), and cucumber (*Cucumis sativus)*. Keratin as fertilizer showed significance ($p < 0.05$) growth at 100x having 0.048 mg/ml keratin protein for root length, shoot length, plant length as compared to other treatment and control (Table 3). 100x (0.048 mg/ml) and 70x (0.068 mg/ml) dilution showed significant ($p < 0.05$) fresh weight and dry weight as compared to other treatments and control (Table 4).

4.10. Keratin as Poultry Feeds

It was observed that keratin as feed gave effective results on the chicken weight. The weight of experimental chicks was greater as compared to control group and significant differences at ($p < 0.05$) was noticed in experimental group with overall weight gain of 67.7% while, it was only 50.9% for the control group (Table 5).

4.11. Liver Function Test

Liver function test was performed for both experimental and control groups and results showed

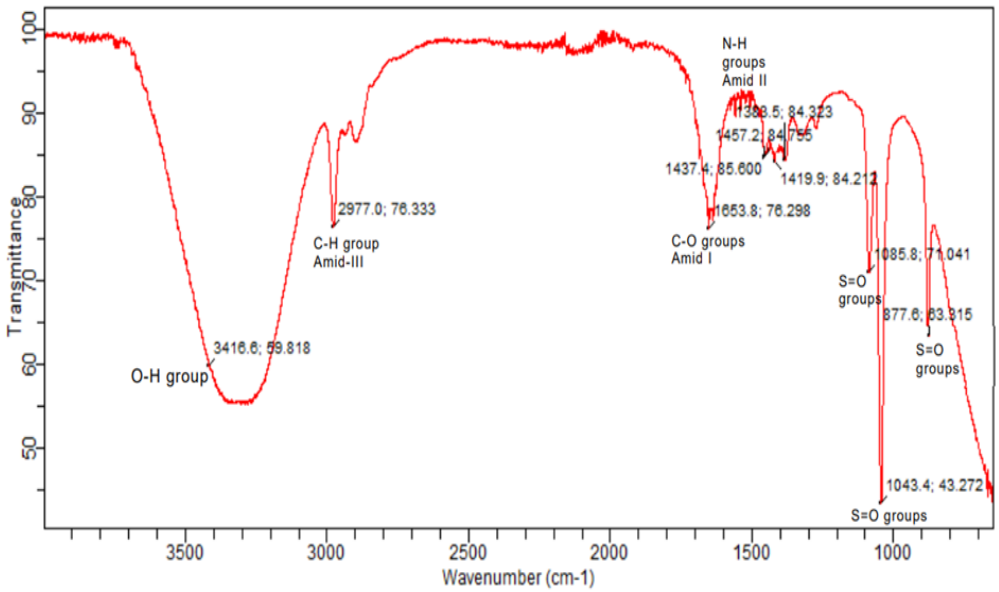

Fig. 7. Fourier Transform Infrared analysis of keratin hydrolysate.

that level of Bilirubin was 0.8 mg/dl for all chicks. While a decrease in ALT (alanine aminotransferase) and AST (aspartate aminotransferase) levels were noticed for experimental groups as compared to control. Levels of ALT and AST were 16.6 U/L and 187.3 U/L for the experimental group and 29.6 U/L and 214.6 U/L for the control group, respectively. While an increase in ALP (alkaline phosphatase) and total blood protein levels were noticed for the experimental group that were 3777.3 U/L and 4.2 g/dl as compared to control group that were 15581 U/L and 4.9 g/dl, respectively. Levels of Albumin and globulin were also determined and increased levels were noticed for experimental group as compared to control. The levels were 1.56 g/dl and 2.63 g/dl for the control while, 1.8 g/dl and 13.9 g/dl for the experimental group for albumin and globulin, respectively. The ratio of albumin/globulin for control on average was 0.59 and for experimental the ratio of albumin/globulin on average was 1.47 (Table 6).

Table 1. Effect of plant microbe interaction on percentage germination and length parameters.

Isolates	Root length (cm)	Shoot length (cm)	Plant length (cm)	Germination %
Tomato (*Solanum lycopersicum)*				
SC1	0.723 ± 0.09	0.78 ± 0.14	2 ± 0.21	46.6 ± 0.70
SC2	1.089 ± 0.22	2.84 ± 0.11	2.86 ± 0.07	33.3 ± 0.98
SC3	0.88 ± 0.04	2.48 ± 0.21	2.68 ± 0.14	66.6 ± 0.77
SC4	1.15 ± 0.22	3.56 ± 0.32	4.6 ± 0.21	66.6 ± 0.91
SC5	1.12 ± 0.04	2.92 ± 0.21	4.34 ± 0.21	66.6 ± 0.63
SC6	1.4 ± 0.13	2.32 ± 0.14	2.6 ± 0.14	40 ± 0.70
SC7	2.64 ± 0.17	3.94 ± 0.21	5.16 ± 0.19	86.6 ± 0.56
SC8	2.36 ± 0.28	3.78 ± 0.12	5.92 ± 0.16	80 ± 0.91
SC9	0.94 ± 0.33	3.24 ± 0.21	4.06 ± 0.20	60 ± 0.98
SC10	0.354 ± 0.21	0.68 ± 0.04	1.29 ± 0.33	53.3 ±0.63
SC11	1.34 ± 0.35	1.38 ± 0.14	2.72 ± 0.11	66.6 ± 0.84
SC12	0.9 ± 0.21	3.52 ± 0.21	4.42 ± 0.14	73.3 ± 0.70
Control	0.414 ± 0.08	0.46 ± 0.05	0.81 ± 0.09	33.3 ± 0.98
LSD for treatment= 1.42		LSD for Isolates= 0.68		
Cucumber (*Cucumis sativus)*				
SC1	1.12 ± 0.16	4.45 ± 0.21	5.57 ± 0.14	53.3 ± 0.70
SC2	1.18 ± 0.14	2.7 ± 0.14	3.88 ± 0.35	40 ± 0.21
SC3	2.97 ± 0.42	4.28 ± 0.28	7.25 ± 0.21	53.3 ± 0.70
SC4	4.02 ± 0.35	4.19 ± 0.21	8.22 ± 0.28	53.3 ± 0.84
SC5	2.91 ± 0.49	4.25 ± 0.35	7.16 ± 0.14	60 ± 0.98
SC6	2.82 ± 0.35	1.52 ± 0.21	4.34 ± 0.21	53.3 ± 0.70
SC7	4.22 ± 0.21	7.84 ± 0.18	12.08 ± 0.28	73.3 ± 0.42
SC8	4.24 ± 0.28	7.6 ± 0.21	11.85 ± 0.14	66.6 ± 0.21
SC9	3.35 ± 0.35	3.32 ± 0.14	6.67 ± 0.21	46.6 ± 0.84
SC10	0.8 ± 0.14	4.96 ± 0.28	5.96 ± 0.28	40 ± 0.28
SC11	3.76 ± 0.11	3.97 ± 0.35	7.73 ± 0.14	53.3 ± 0.21
SC12	3.58 ± 0.21	6.36 ± 0.21	9.94 ± 0.28	60 ± 0.35
Control	1.61 ± 0.21	2.69 ± 0.14	4.3 ± 0.21	40 ± 0.28
LSD for treatment 1.88		LSD for Isolates 0.90		

Table 2. Effect of plant microbe interaction on fresh weight and dry weight.

Isolates	Fresh weight (g)	Dry weight (g)
Tomato (*Solanum lycopersicum)*		
SC1	0.4729 ± 0.21	0.0332 ± 0.01
SC2	0.2749 ± 0.14	0.0275 ± 0.03
SC3	0.6688 ± 0.21	0.0561 ± 0.02
SC4	0.7236 ± 0.14	0.1259 ± 0.14
SC5	0.7759 ± 0.35	0.2955 ± 0.07
SC6	0.8224 ± 0.14	0.3952 ± 0.02
SC7	0.8517 ± 0.35	0.5948 ± 0.04
SC8	0.9076 ± 0.28	0.743 ± 0.14
SC9	0.7935 ± 0.14	0.453 ± 0.28
SC10	0.3758 ± 0.28	0.002 ± 0.001
SC11	0.8636 ± 0.42	0.45 ± 0.07
SC12	0.7049 ± 0.14	0.252 ± 0.06
Control	0.2679 ± 0.12	0.0133 ± 0.003
LSD for treatment 0.19		LSD for Isolates 0.08
Cucumber (*Cucumis sativus)*		
SC1	0.3 ± 0.28	0.024 ± 0.02
SC2	0.34 ± 0.14	0.02 ± 0.04
SC3	0.32 ± 0.42	0.02 ± 0.11
SC4	0.46 ± 0.35	0.027 ± 0.03
SC5	0.86 ± 0.16	0.009 ± 0.007
SC6	0.53 ± 0.21	0.013 ± 0.004
SC7	0.81 ± 0.16	0.036 ± 0.04
SC8	1.46 ± 0.21	0.09 ± 0.03
SC9	0.37 ± 0.14	0.008 ± 0.007
SC10	0.28 ± 0.28	0.008 ± 0.003
SC11	0.27 ± 0.14	0.008 ± 0.004
SC12	0.27 ± 0.35	0.005 ± 0.002
Control	0.06 ± 0.02	0.03 ± 0.04
LSD for treatment 0.50		LSD for Isolates 0.2

Table 3. Effect of Keratin hydrolysate as fertilizer on root, shoot, and plant length.

Dilutions	Root length (cm)	Shoot length (cm)	Plant length (cm)
Tomato (*Solanum lycopersicum)*			
10x	1.2 ± 0.14	0.62 ± 0.14	1.82 ± 0.22
20x	0.56 ± 0.15	1.7 ± 0.07	2.26 ± 0.28
30x	1.28 ± 016	2.28 ± 0.21	3.56 ± 0.14
50x	1.8 ± 0.14	2.44 ± 0.28	4.24 ± 0.16
70x	2.2 ± 0.14	2.22 ± 0.15	4.42 ± 0.15
100x	5.2 ± 0.07	3.43 ± 0.28	8.9 ± 0.28
Control	0.55 ± 0.07	1.72 ± 0.14	2.27 ± 0.19
LSD for treatment 1.54			
Pea (*Pisum sativum)*			
10x	1.42 ± 0.14	2.36 ± 0.14	3.78 ± 0.15
20x	1.45 ± 0.21	1.76 ± 0.28	3.21 ± 0.21
30x	1.33 ± 0.21	3.18 ± 0.35	4.51 ± 0.28
50x	1.68 ±0.15	3.48 ± 0.28	5.14 ± 0.25
70x	1.81 ± 0.21	4.89 ± 0.21	6.69 ± 0.28
100x	3.05 ± 0.12	5.38 ± 0. 35	8.45 ± 0.20
Control	1.13 ± 0.09	1.3 ± 0.14	2.43 ± 0.28
LSD for treatment 1.37			
Cucumber (*Cucumis sativus)*			
10x	1.76 ± 0.16	0.61 ± 0.14	2.37 ± 0.35
20x	1.78 ± 0.26	0.92 ± 0.57	2.7 ± 0.21
30x	2.07 ± 0.04	2.4 ± 0.28	4.29 ± 0.21
50x	4.3 ± 0.28	2.35 ± 0.35	5.16 ± 0.42
70x	5.61 ±0.20	3.74 ± 0.18	9.35 ± 0.14
100x	6.2 ± 0.21	6.5 ± 0.35	12.7 ± 0.21
Control	1.1 ± 0.35	2.4 ±0.31	3.5 ± 0.28
LSD for treatment 2.2			

10x (keratin 0.48mg/ml) 20x (024mg/ml) 30x (0.09mg/ml) 50x (0.09 mg/ml) 70x (0.068mg/ml), 100x (0.048 mg/ml)

4.12. Histopathology Test of Liver Chicken

The slides of stained liver tissue were observed in a light microscope under 100X lens, Figure 8 shows the histopathology of the chicken in control group; while Figure 9 presents the histopathology of liver chicken in experimental group. Keratin seemed to have no degenerative effect on the liver as hepatocytes were healthy. The cellular structure of hepatocytes was found same in both control and experimental groups except difference in cell size that was 24 µm in experimental and 26 µm in control group. The slight reduction in liver cell size in keratin-fed chickens suggests improved nutrient utilization, supporting healthy growth without liver stress.

5. DISCUSSION

Keratin present in feathers is highly difficult to degrade but there are some microorganisms that produce keratinase enzymes, which could

Table 4. Effect of Keratin hydrolysate as fertilizer on fresh and dry weight of different plants.

Dilutions	Fresh weight (g)	Dry weight (g)
Tomato (*Solanum lycopersicum)*		
10x	0.0299 ± 0.01	0.0243 ± 0.02
20x	0.0379 ± 0.02	0.018 ± 0.04
30x	0.0858 ± 0.01	0.0467 ± 0.03
50x	0.3045 ± 0.07	0.0869 ± 0.01
70x	0.514 ± 0.21	0.0965 ± 0.02
100x	0.9032 ± 0.07	0.3964 ± 0.07
Control	0.2729 ± 0.21	0.0188 ± 0.02
LSD for treatment 0.34		
Pea (*Pisum sativum)*		
10x	0.12 ± 0.05	0.009 ± 0.002
20x	0.11 ± 006	0.011 ± 0.004
30x	0.07 ± 0.02	0.013 ± 0.004
50x	0.11 ± 0.06	0.024 ± 0.007
70x	0.15 ± 0.04	0.022 ± 0.009
100x	0.15 ± 0.01	0.049 ±0.01
Control	0.06 ± 0.02	0.007 ± 0.005
LSD for treatment 0.04		
Cucumber (*Cucumis sativus)*		
10x	0.32 ± 0.14	0.022 ± 0.02
20x	0.32 ± 0.21	0.02 ± 0.02
30x	0.37 ± 0.30	0.01 ± 0.02
50x	0.49 ± 0.14	0.033 ± 0.01
70x	0.54 ± 0.21	0.031 ± 0.02
100x	0.76 ± 0.13	0.018 ± 0.02
Control	0.06 ± 0.04	0.08 ± 0.03
LSD for treatment 0.33		
10x (keratin 0.48mg/ml) 20x (024mg/ml) 30x (0.09mg/ml) 50x (0.09 mg/ml) 70x (0.068mg/ml), 100x (0.048 mg/ml)		

efficiently degrade this resistant keratin [20]. In this study, the bacterial isolates were isolated from the soil of poultry dump site, purified by streaking and screened on skim milk agar for determining proteolytic activity. Similar methods for isolation

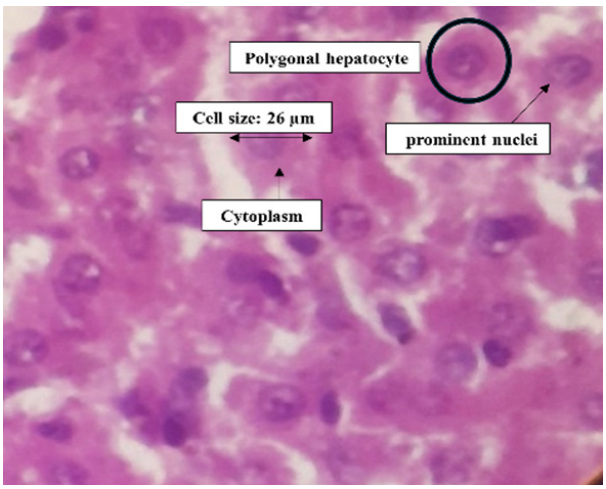

Fig. 8. Histopathology of the chicken in control group.

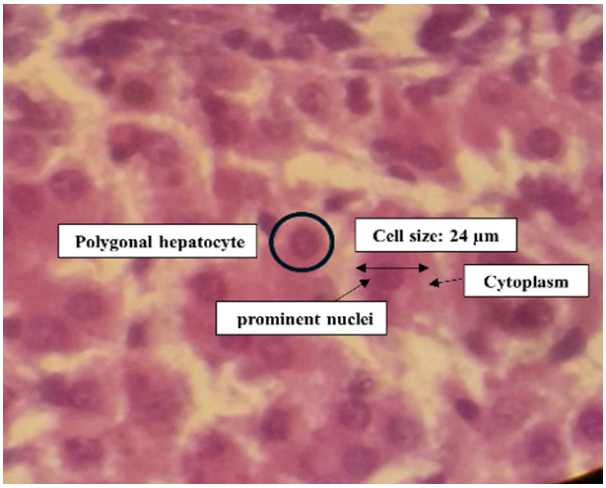

Fig. 9. Histopathology of liver chicken in experimental group.

and screening from poultry dump site were investigated previously [6, 20, 21]. The powder of *Gallus gallus domesticus* feathers was white color with powder yield 50%. By identification based on morphological, cultural and microscopic characteristics, the identity of the isolates was determined to be *Bacillus spp*. The recorded results are in line with previous findings about keratinolytic organisms which are found to *Bacillus spp.* [3, 12, 22-25].

Taxonomic affinities based on 16S ribosomal RNA gene sequence were analyzed using commercial service of gene sequencing (IBM) due to its reliable and trustworthy results. Results of 16S ribosomal RNA gene sequence were submitted in gene bank NCBI. Results showed bacterial

Table 5. Effect of Keratin hydrolysate as feed on chicken weight.

	Control group (without Keratin feed)			Experimental group (with Keratin feed)		
Sr. no.	**Initial weight (g)**	**Final weight (g)**	**% increase in weight (g)**	**Initial weight (g)**	**Final weight (g)**	**% increase in weight (g)**
Chick 1	76	94	23.6	90	148	64.4
Chick 2	51	77	50.9	93	156	67.7
Chick 3	76	86	13.1	76	101	32.8
LSD for treatments = 19.44, LSD for chicks = 15.87						

Table 6. Levels of different parrameters in liver function test.

Sr. no.	**BILI mg/dl**	**ALT U/L**	**AST U/L**	**ALP U/L**	**TP g/dl**	**ALB g/dl**	**GLOB g/dl**	**A/G**
Control chicks	0.53 ± 0.12	29.6 ± 3.37	214.6 ± 4.01	15581 ± 775.3	4.2 ± 0.05	1.56 ± 0.05	2.63 ± 0.03	0.59 ± 0.02
Experimental chicks	0.53 ± 0.12	16.6 ± 2.5	187.3 ± 4.28	3777.3 ± 591.8	4.9 ± 0.11	1.8 ± 0.17	13.9 ± 0.15	1.47 ± 0.01

BILI (bilirubin), ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), TP (total protein), ALB (albumin), GLOB (globulin), A/G (albumin/globulin).

homology with *Pseudomonas aeruginosa* bacterial identification on basis of 16S ribosomal RNA gene sequence is reliable for the identification of bacterial isolates. Comparison of 16S ribosomal RNA gene sequence revealed that these bacteria are diverse and predominated in ecological nations.

The keratinase enzyme production from *Pseudomonas aeruginosa* from 122 U/ml to 150 U/ml in organic and inorganic conditions was reported by Moonnee *et al.* [26]. The microbial strains which are isolated from waste of poultry and are best for the chicken feathers degradation are *Bacillus sp., Kocuria sp., Pseudomonas sp., and Fervidobacterium sp.* [27].

Effects of physiological conditions, pH and temperature, on bacterial growth were checked and results showed that there was a general trend of significant ($p < 0.05$) increase in cell densities at pH 9.0 and temperature 45 °C as compared to pH 5.0 and 7.0 and temperatures 25 ℃ and 37 ℃. Feather substrate degradation was established to be correlated with significant ($p < 0.05$) rise in pH of the medium towards alkalinity, thus giving an indication of the efficient degradation. These observations are in line with previous findings wherein keratinolytic bacteria have been reported to show optimal growth at thermophilic temperatures [28-30]. The degradation duration decreased at 45 ℃ as it was completed in 2 weeks instead of 4 weeks like at 37 ℃.

The keratin protein concentration in chicken feathers was found to be significant ($p < 0.05$) at pH 9.0 and temperature 45 ℃ which was 4.8 mg/ml and 5.5 mg/ml, respectively. There was an increase in soluble keratin protein content with an increase in pH of medium [30]. Sivakumar and Raveendran [32] estimated the keratin protein to be 414 µl/ml from chicken feathers degraded by *B. stubtilis*. An estimated keratin protein from chicken feathers degraded by *B. licheniformis* to be 8.28 mg/ml at 40 °C and pH 10.0 was reported in a study [33].

All 12 bacterial isolates obtained from *Gallus gallus domesticus* showed positive biofilm formation in 4 days at a temperature of 45 ℃ and pH 9.0. The biofilm formation by keratinolytic microbe increases the amount of keratin in extraction and promotes the degradation rate [34].

The FTIR analysis of keratin hydrolysate was observed between 4000-700 cm^{-1} wavelengths. O-H stretching absorption peak range was 3500-3100 cm^{-1}. Amid group III having C-H stretching was indicated by absorption peaks 3000-2900 cm^{-1}. Amid group I have C=O stretching was indicated by absorption

peaks 1450-1400 cm^{-1}. Amid group II having N-H bending was indicated by absorption peaks 1450-1400 cm^{-1}. Cysteine, S=O, stretching was observed at peaks ranging from 1100-800 cm^{-1}. The results of this study are in accordance with observations made by previous researchers [25, 33-37], who also found the FTIR spectrum range to be 4000-400 cm^{-1} and similar peaks for the functional groups.

Plant microbe interaction indicated that there was a general trend of significant ($p < 0.05$) increase in germination and growth of tomato and cucumber seeds as compared to control. *B. subtilis PF1* and metabolites that were released due to degradation of chicken feathers can be applied as nitrogen fertilizer for plants [38]. While *S. maltophilia R13* could be used as biocontrol agent and could also affect the fertilization of soil [39]. Similarly, *Bacillus cereus* could not only degrade chicken feathers but also improve plant growth development and is an effective plant growth promoting rhizo-bacteria [40].

Keratin as fertilizer showed significance ($p < 0.05$) growth at 100x having 0.048 mg/ml keratin protein for root length, shoot length, plant length fresh and dry weight as compared to other treatment and control. The optimal keratin concentration in feathers hydrolysate for seed germination is 0.1 mg/ml or below 0.1 mg/ml [41]. The compost was prepared from degraded chicken feathers and found out that it could be an economic source of nitrogen fertilizer [42]. The application of chicken feather hydrolysate to gram seeds resulted in early germination of seeds and higher growth of plant as compared to control [43].

Keratin hydrolysate increases the body weight of broiler chicken up to 9-10% as compared to fishmeal. The inclusion of feather meals in feed of broiler chicken had no negative effect on birds and it also reduces feed cost [44]. The increase in live weight of chicken to be 8.6% ($p < 0.001$) as compared to control [45]. It also increases the productivity of broiler but also higher antioxidant meat. To sum up, the present findings showed the efficacy of keratin degrading bacteria in poultry waste utilization into value added products.

The parameters of liver function tests can be used to determine cell injury of response to such an injury [46]. Blood activity of *Gallus gallus domesticus* can be determined by liver function tests [47].

The bilirubin was 0.8 mg/dl for both experimental and control chicks. The ALT (alanine aminotransferase) level was reduced for experimental setup as compared to control setup. For experimental the ALT level on average was 16.6 U/L while the control had 29.6 U/L. The AST (aspartate aminotransferase) level was reduced for experimental setup as compared to control setup. For experimental the AST level on average was 187.3 U/L while the controlled had 214.6 U/L. The ALP (alkaline phosphatase) level was reduced for experimental setup as compared to control setup. For experimental the ALP level on average was 3777.3 U/L while the control had 15581 U/L. Total protein was increased for experimental setup as compared to control setup. For experimental the total protein level on average was 4.9 g/dl while the control had 4.2 g/dl. The albumin was increased for experimental setup as compared to control setup. For experimental the albumin level on average was 1.8 g/dl while the control had 1.56 g/dl. Globulin was increased for experimental setup as compared to control setup. For experimental, the globulin level on average was 13.9 g/dl while the control had 2.63 g/dl. The ratio of albumin/globulin was increased for experimental setup as compared to control setup. For experimental the albumin/globulin on average ratio was 1.47 while the control had 0.59.

The normal values of level of ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), Total protein, Albumin, Globulin and Albumin/ Globulin ration in chicken were described as liver of chicken is 0.9 µg/kg/min [48]. The normal range of ALT in chicken is 15.3-55.3 U/L [49]. The AST normal range for chicken is 70-220 U/L and ALP normal range for chicken is 568-8831 U/L [50]. The normal range of total protein to be 33-55 g/l, albumin to be 13-28 g/L and globulin to be 15-41 g/l reported for chicken [51]. [In another study the albumin/globulin ratio for chicken from day 4 to day 46 was reported as 0.59-0.85 [52] and the albumin/globulin ratio for chicken from day 14 to day 42 was also depicted as 1.50-1.72 [53].

The histopathology results of chick liver indicated that keratin had no degenerative effect on the liver as hepatocytes were healthy. Xu *et al*. [54] found that nanoparticles of keratin are useful for the delivery of drugs to the kidney and liver. The use of chicken feathers for the formation of hydrogel

based on keratin also reported by Tang *et al*. [55]. These results showed keratin to be an effective drug carrier and had good hemostatic effect on tail and liver injury of mouse. Similarly, studies with nanoparticles prepared from keratin of chicken feathers showed an increased rate in coagulation in liver scratch model exhibiting their effectiveness in bio medical application [56, 57].

6. CONCLUSIONS

Avian feathers can be degraded by *Bacillus* strains isolated from soil at optimum temperature (45 °C) and pH (9), particularly within 3 weeks. This can help with the rapid removal of feathers' waste that is coming from the poultry industry. The keratin hydrolysate obtained as a result could be a cheaper source of fertilizer and an alternative of chemical fertilizers as it has potential for the growth of plants. The keratin hydrolysate can also be used as feed additive, and it helped for the better productivity of chickens in poultry industry as an increase in body weight and healthier chicken were observed during the study who were fed with keratin hydrolysate.

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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