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PREFACE

This book covers key areas of biological science. The contributions by the authors include humoral structures, inter-organic and functional synchronization, informational principle, synergetics, quantum mechanics, classical physics, quantum cryptography, Einstein's theory, water regulates homeostasis, dynamic equilibrium, oscillatory homeostasis processes, biopolymer conformation, quantum dots, information processing, supramolecular systems, wave-particle, bovine horn core carcinoma, squamous cell carcinoma of horn, cytokeratin, non keratinizing type tumours, celeastralean plexus, taxonomy, iron deficiency anemia, optical coherence tomography, neurometabolism, physiologic needs, coronary heart disease, edible crustaceans, nutritional values, lipids and fatty acids, nutritional composition, human hepatocellular carcinoma, historical significance, community empowerment, genome editing, Cas9 protein, guide RNA, CRISPR technology, cottonseed meal, rice husk, cholecalciferol supplementation, anti-nutritional factors. This book contains various materials suitable for students, researchers, and academicians in the field of biological science.

Chapter 1
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Dissipative Regulation in a Human Body

Igor Orzhelskyi [a++], Andrey Kuznetsov [b#] and Elena Van Dijk [c++*]

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ABSTRACT

Inter-organic and functional synchronization and dissipative regulation are carried out through humoral structures (blood, lymph). Neuronal structures also function with the participation of dissipative regulation. The functioning of a living body, maintaining the dynamic stability of its homeostasis, is provided by regulatory systems. Traditionally, such systems include the nervous and humoral systems of regulation. The third regulatory system of the body, which provides communication in the body, distance interactions according to the "informational" principle, is not considered. For this reason, the understanding of the law of nature – “substance-energy-information”, uniting the foundations of any matter, is very limited in biology. Synergetics is a science that explores the self-organization of complex systems and processes, combining different interdisciplinary areas of science. Moreover, these complex systems and processes are dissipative, i.e., exchange with the environment substance, energy, information. Therefore, the rules and postulates of Synergetics, as a science of the self-organization of matter, in the understanding of modern scientists are far from real processes occurring in matter "here and now." This article provides a summary of the basic rules and principles of the interaction of Matter, Energy and Information, which is the basis of the third regulation mechanism in the human body.

Keywords: Synergetics; dissipative regulation; signal molecules; humoral system.

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1. INTRODUCTION

Dissipative processes of self-organization occur in both living and non-living matter. The same laws govern living and non-living matter, but the mechanism of their manifestation is different. The distinctive feature of living matter is “functional information,” which is an attribute characteristic only of living matter [1]. The functioning of a living body, maintaining the dynamic stability of its homeostasis, is provided by regulatory systems. Traditionally, such systems include the nervous and humoral systems of regulation. These systems are deeply integrated. Therefore, they are often even combined into a common name for neurohumoral regulation. Regulatory signals that play a communication role directly in these systems include: in the humoral system - signal molecules (biochemicals), and in the nervous system - nerve impulses (electrical signals). The nervous system provides a reasonably quick regulation. The humoral system is more inert than the nervous system.

2. REGULATION MECHANISMS IN THE HUMAN BODY

2.1 A Review of Existing Understandings of Regulation

Many scientific studies and the results obtained indicate that the regulatory principles and mechanisms of these two systems do not adequately describe the properties and processes that occur in living organisms [1a,2].

In the historical context, the description of the dynamic features and methods of the matter reduced to the determination of hierarchical structural interacting units and the result of their structural transformations with the participation of energy [3,4]. The third, not the only, but an essential participant in such changes, providing interaction, “information,” as a rule, is not considered. In the best case, the role of “information” is attributed exclusively to the structural components of matter itself, for exemple, such as various hormones, enzymes and others.

It is at this stage that there is a substitution, and perhaps misunderstanding, of the actual in-depth content and role of "information" in such interactions and transformations. In this regard, the part and significance of the trinity of “substance-energy- information,” which together determine the law of conservation of energy, remain blurred and limited in biology [5]. Therefore, the rules and postulates of Synergetics, as the sciences of the self-organization of matter, are perceived as something abstract, not directly related to the real processes taking place in the matter “here and now” [6]. Nevertheless, the structural and functional conditioning of the transformation, the changed properties of matter, are considered apparent.

2.2 The Effects of “Information” as a Regulatory Mechanisms

The lack of understanding in biology and medicine of the "object of information reception" and the role from the impact of the material "subject" (factor) of information, does not allow to define the very concept of “Information”. The

current situation also does not allow us to accept the fact that “Information” is an indispensable and an obligatory participant in any interaction and transformation in the matter. This state of affairs in the natural sciences (biology, medicine and others), has existed for a long time, and does not find not only an appropriate solution, but even the need to pose a question to resolve it. At the same time, in other areas of science, which are far from biology and medicine (such as hydrodynamics, chemical transformations and reactions of complex molecules, and even social phenomena, etc.), all these processes of self-organization and, “information” associated with them, is one of the critical factors for for analysis and research of complex processes in thease scientific areas.

Here, self-organization processes are considered as processes of transition from a less ordered state to a more ordered one based on the “principle of collectivity”. The number of participants in the system (process), their collective, coordinated behavior and interaction are reflected in the formation of new (acquired) connections, and the properties of the whole system. Moreover, the processes themselves, the action of the collective participants (subjects) of the process, obey mathematical patterns. Cooperative participants in the new dynamic state, with minimal energy costs, show increased consistency (synchronism) and sensitivity (sensitization) as a reaction to external influences.

Synergetics is a science that explores the self-organization of complex systems and processes, combining different interdisciplinary areas of science. Moreover, these complex systems and processes are dissipative, i.e., exchange with the environment substance, energy, information. They are in a state of dynamic chaos, and for the most part, the processes in them are stochastic (random), non- deterministic. However, these systems in their behavior reveal correlation relationships and patterns that obey the well-known both physical and mathematical laws.

An essential components of the dynamic state and processes in dissipative systems, is the medium, which not only combines the elements of the system, but also, to a large extent, determines the properties and evolution of this dissipative system.

2.3 Dissipative Systems in the Regulatory Processes of the Body

The most important feature of the medium at the dissipative systems is its constant fluctuation (oscillation, vibration). Processes, system elements in which vibrations and fluctuations (variability) are present- have self-synchronization and self-organization. Without a unifying framework (medium) uniting these processes, synchronization would be impossible.

At the same time, all together these factors: a change in the properties of the medium; the energy and information, introduced into the dissipative system, can disrupt the current synchronization and self- organization existing in it. Accordingly, the dissipative system may lose the properties that were inherent in it, as a single coordinated dynamic object.

The modern challenge of synergy is set to clarify of the patterns of behavior of dissipative systems across microcosm.

An essential stage in this direction was the study and discovery of the properties of supramolecular systems that are part of complex dissipative systems of the living matter [7]. Such systems include genome molecules, organelles, tissue cells, where the medium that unites them is an indispensable participant.

It is well-known that these supramolecular systems have highly specific properties and, in particular, can recognize each other, move, and carry out various transformations in interaction with other molecules. The belief is that supramolecular systems are a bridge between the living and nonliving matter [8]. A group of scientists - Donald James Crum, Jean Marie Len and Charles Pedersen were awarded the Nobel Prize in 1987 for the “Development and use of molecules with structurally specific interactions of high selectivity.” Nature used supramolecular systems to create biological systems.

The question arises: what (!) in these complex molecular systems, with specific properties, is the “force point”, thereby the “receptor”, that can respond to fluctuating medium conditions and interaction of similar molecules (system elements)?

When representing a molecule as a dynamic, spatially organized chemical structure, attention should also be addressed to its internal conformational molecular components, such as ionic bonds between atoms and the angles at which these atoms are located relative to each other. It is known that with the same chemical composition of molecules with various properties of the environment and external influences, these ionic bonds and angles can vary [9-12]. Moreover, with a change in the conformation of the molecule, the properties of this molecule also change. An indispensable participant in such changes in living media is various derivatives of water molecules involved in the hydration of many molecules, including proteins. However, it is equally crucial that ionic bonds and angles in a molecule can not only change their sizes but also, through the medium of their location, distantly synchronize with similar ionic bonds and angles in other similar molecules [13]. With such synchronization, a dissipative system arises, as a new “collective - unified” system, but with newly acquired properties that are inherent to this entire self-organized system.

Among these acquired properties it is necessary to single out the minimum energy costs for interaction, reaction, stabilizing the system, which is manifested in increased consistency (synchronism) and sensitivity, as a reaction to external influences.

The formed new state of the system co-occurs with the participation of all participants in the system, its components. Such a new state of the system carries a new regulatory (informational) signal that is complementary meaningful already for other levels of cascade (hierarchical) interaction and regulation. Moreover, the dissipative properties of the regulatory signal formed equally for

the entire dissipative system, with minimal external influences on it. Furthermore, the dissipative system itself performs the functions of a “Receiver,” a "Converter" (carrying out a change in the information density of the regulatory signal) and a “Repeater.”

Consider an example of intracellular regulation. At this level, regulation is not only based on the principle of “receptor-ligand” interaction, but also by the principle of simultaneous relay of the ligand signal (for example, any hormone) to all the same type of cell receptors. And this “Repeater” is a dissipative medium “receptor-ligand” and extracellular matrix (ECM). Moreover, in this process, humoral and dissipative regulation act together. Violation of dissipative regulation inevitably leads to a change in humoral regulation on the principle of positive or negative feedback.

Moreover, in the same medium (EMC) at the same time can operate without interfering with each other, a large number of dissipative systems. Diversity, or in other words, the principle of "no intervention" is based on the difference of the polarization properties of dissipative systems.

It is essential to answer the question: what is the basis for the perception, reading, dissemination, and destruction of regulatory information continually coming from the outside?

The extracellular matrix, however, like the protoplasmic (intracellular matrix), is a complexly organized anisotropic molecular structure. The matrix can stay in its two primary states - sol or gel [14].

Sol is a minimally ordered matrix phase. It is in this phase that the maximum entropy in the system is observed (maximum chaos, disconnected bonds). In this phase, the mechanisms of diffusion transfer and interactions are realized [14].

The gel is formed by the binding of protein molecules or other colloidal structures to large quantities of ionic derivatives of water molecules. The gel phase is a highly structurally organized phase. It is in this phase that the ECM has minimal entropy (minimal chaos, high consistency, or, in other words, synchronization of connections). The system responds to minimal, in terms of energy, external fluctuations. It is in the gel phase that the mechanism of dissipative regulation and interaction is realized. This means that in the gel phase, the dissipative system converts and transmits a regulatory information signal to all receptors, complementary to this signal. Thus, the perception, reading, distribution, and, in general, relaying of the regulatory signal occurs in the gel phase. In the sol phase, the information state of the dissipative system is reset. The self-oscillatory nature of the “sol-gel transition” rhythm ensures the cascading nature of informational regulatory interactions.

The ratio of the sol-gel transition rhythms changes under the influence of various factors, including pathological processes affecting the ECM of a particular tissue. This circumstance makes a significant contribution to the dynamics and nature of the development of diseases.

It is important to note that the transfer of regulatory information, according to the cascade principle, is also carried out between complementary dissipative systems. Dissipative ECM-information is translated into interconnected dissipative system receptors of cells. If the ECM, as a "Transmitter" and a "Repeater", functions as efficiently as possible, it means then “Receiver effective antenna area” is required less. This role of “Antenna” on the cell membrane is collectively performed by the same type of cell-receptors. Moreover, their synchronization ensures maximum efficiency of the “receiving antenna”. If the receptors, for whatever reason, are not synchronized, to guarantee the sufficiency of the received signal, need a more significant number of such receptors and (or) a stronger (in terms of information density) external signal from the EMC is necessary.

3. CONCLUDING REMARKS

Inter-organic and functional synchronization and dissipative regulation are carried out through humoral structures (blood, lymph). Neuronal structures also function with the participation of dissipative regulation. So, a neuronal electrical impulse overcomes the synaptic gap in the “sol-phase” and the “gel-phase” for its electrical impulse, an “insulator”. The state of "sol-gel transitions" in neuronal processes is significantly reflected in a variety of conditions and operations of a living organism.

Thus, the system of dissipative regulation is essential, along with the neuronal and humoral systems of regulation. Moreover, a violation of the physiological mechanisms of dissipative regulation is necessarily reflected in the other two, the neuronal and humoral systems of regulation.

A reasonable question arises the consideration of approaches and principles, the creation and implementation of specific means for physiological normalization and restoration of dissipative regulation, along with the humoral and neuronal systems. We will continue to consider this issue in future publications.

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Chapter 2
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The Quantum Symphony of Life: Insights into Biological Systems

Igor Orzhelskyi [a++], Andrey Kuznetsov [b#] and Elena Van Dijk [c++*]

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ABSTRACT

The study effectively introduces and explores complex concepts in condensed matter physics, quantum mechanics and their applications in various systems, including biological ones. The connection between quantum principles and real-world technologies, such as quantum cryptography and computers, adds practical relevance. Integrating ERI technology in the context of quantum mechanisms provides a bridge between theoretical concepts and potential practical applications in healthcare. The functioning of a biological systems, maintaining the dynamic stability of its homeostasis, is provided by regulatory systems. Traditionally, such systems include the nervous and humoral systems of regulation. The third regulatory system of the body, which provides communication in the body, distance interactions according to the “informational” principle, is not considered. For this reason, the understanding of the law of nature – “substance-energy-information”, uniting the foundations of any matter, is very limited in biology. The significant synchronization of oscillations of their structures and processes allows these systems to effectively respond to weak regulatory signals and to form their own response signals of sufficient intensity in the system of communication links and interactions. This article provides a summary of the basic rules and principles of the interaction of Matter, Energy and Information from a quantum perspective.

Keywords: ERI technology; quantum principles; quantum particles; oscillations.

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1. INTRODUCTION

1.1 Condensed State of Matter, Non-Locality and Information

One of the important topics in the protuberant area of physics is condensed matter physics and it broadly encompasses the microscopic and macroscopic physical properties of materials [1]. Condensed matter physics is a large branch of physics that studies the behavior of complex systems (i.e., systems with a large number of degrees of freedom) and strong bonds between particles in such systems. One essential characteristic of quantum systems is that, regardless of the distance between particles, the dynamics of their behavior as a result of external influences are instantly reflected on the system as a whole and on each particle individually. As long as there is a condensed state of matter and information between quantum biological things, this mechanism is non-local in nature. Such a mechanism of interaction of quantum particles is called as non-local because quantum particles are not located at any point in space but they are represented as a waves. Non-locality is a property associated with the transmission of information [1a–6]. It is characterized by the immediate reaction of particles (or systems) to the change of parameters of one of them which is simmultanously changing the parameters of the others, no matter how far the particles (or systems) are from each other. As early as 1935 was formulated so called Einstein´s paradox. It stated that “unlike the principles of the theory of relativity, quantum equations point to the immediate connection of all parts of space as a whole.”

1.2 The Limits of Applicability within the Laws of Classical and Quantum Physics

1.2.1 Description of effects and properties of macro-, micro- and biological objects

Classical physics considers radiation as an emission of electromagnetic waves with accelerated moving electric charges. Classical theory explained many characteristics of radiation processes, but could not give a satisfactory description of number of events particularly thermal radiation of organisms, microsystems (atoms and molecules). Such a description turned out to be possible only within the framework of the quantum theory of radiation, which showed that radiation is the production of photons during the change of state of the quantum system (for example atoms). Quantum theory, having penetrated more deeply into the nature of radiation, simultaneously indicates the limits of applicability of the classical theory. It provides a very good approximation for describing radiation, remaining, for example, the theoretical basis of radio engineering.

Physicists have found out that quantum mechanics practically does not work in the macrocosm and only applies to objects no larger than atoms and charged particles (molecules), since for larger objects the action of the force of gravity is manifested, which is reflected in the inhomogeneous deceleration of time flow

rate. According to Einstein's theory of general relativity, time flows non-uniformly in the presence of gravitational fields. The stronger the field, the slower time flow rate. Under gravity, coherent connections between objects of large systems are destroyed and begin to depend on distance of these objects at the current time in relation to the center of mass. The behavior of most objects, systems of the world visible to us can be described using simple laws of classical physics, without taking into account the possible influence of quantum factors, which is suppressed by gravity and associated with its phenomena.

However, in many cases, we are able to observe the manifestation of quantum effects in systems consisting of subatomic, atomic particles charged particles (molecules), photons and even cells. In such systems, the non-local nature of interaction can be manifested between its objects. Currently, the nonlocality of quantum objects is a proven experimental fact many times over. For example lasers operating at the same frequency exhibit interaction with each other for no apparent reason. Nowadays the property of photon non-locality is used in the creation of quantum cryptographic systems. The main work resource of such systems are entangled (connected) states of photons and their instant non-local connection (quantum correlations), which allows providing absolute protection of information from unauthorized access. The connection between entangled photons is not just "superluminal", namely the infinite, instantaneous, but in this case it is used not to transmit information, but to control the security of the communication channel. When accessing the transmitted information "from the outside", the coherence of photons (quantum entanglement) is disrupted. Such systems use a fiber optic communication channel.

Scientists are currently working on a quantum computer. Entanglement between qubits (a quantum computer uses quantum bits – qubits) and "entanglement" is nothing more than a correlation, non-local connection of two or more objects separated in space - this is a necessary condition for a quantum computer to work, this is a key factor responsible for quantum parallelism and determines the advantage of a quantum computer over a conventional one. The quantum communication channel, in fact, unites the source and the successor (transmitter and receiver) of information into a single unit with individual degrees of freedom.

1.2.2 Non-locality in biological systems

Such a deeper distant interaction mechanism is also propagating at the quantum level of biological structures and systems Since the 60s. In Novosibirsk, Academician V.P. Kaznacheev and his colleagues conducted studies that confirmed the presence of distant intercellular interactions. In the course of these studies, the so-called mirror cytopathic effect was discovered, when cultures of living cells and tissues, hermetically separated by quartz glass, exchange regulatory wave information related to the functions of the genetic apparatus. It was shown that external information has a correlating effect on the cell, and the mechanism of transmission of the control information signal and intercellular interactions are non-local.

In the macrocosm, any movement is continuous, and therefore has a trajectory of movement. There is no concept of trajectory in quantum mechanics particles. The location of a quantum object can be characterized only by the probability function of finding a given object in a certain region of space. In this regard, the description of the processes of quantum objects and the mechanisms of their interaction can be assessed only as probabilistic. The processes and states of biological systems considered as nondeterministic, stochastic, nonlinear, can be described on the basis of probability functions and fully correspond to the approaches in describing quantum objects and systems. Note that the brain also works in accordance with the principle of "non-locality". For the manifestation of the effect of nonlocal interaction for quantum systems, a necessary condition is to maintain a high degree of coherence (synchronization) between the particles that form these systems - atoms, molecules, cells [7–9].

The significant synchronization of oscillations of their structures and processes allows these systems to effectively respond to weak regulatory signals and to form their own response signals of sufficient intensity in the system of communication links and interactions. The system acquires the properties of a condensed state of matter. This also reveals the property of additivity from their cooperative coordinated functioning. We have already noted that under normal conditions of physiological processes, the normal state of biological tissues and environments is observed relative significant synchronicity between the structures that form these biological systems. With pathologies and conditions preceeding it, a similar synchronous connection is violated.

1.2.3 Analytical cloud system ERI

The practical output of our research represents the Analytical Cloud System ERI. The task of compensatory correction in ERI technology is purposefully, in many cases with a high degree of selectivity to restore broken synchronization of structures of systems and processes. The quantum nature of this mechanism of action is manifested in the technical implementation of a regulatory signal based on the formation of a dynamic electric field strength corresponding to a certain probability function, complementary to such a system or process. If the amplitudes of the probability density of the regulatory signal and the process coincide in phase, then they enhance each other, folding, and if they are in antiphase, then, on the contrary, extinguish each other, decreasing.

2. CONCLUSION

The quantum mechanism of the regulatory impact of ERI in the first place aims at restoring non-local communication in individual quantum systems of the body by restoring their coherent (synchronous) state. Due to this the information links and regulatory processes are restored between structural elements, both within these systems, and subsequently between other systems as well [10–12].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 3
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Significance of Water for Foundation of Dissipative Regulation of a Living Organism

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ABSTRACT

Water regulates homeostasis and supports metabolism by maintaining the balance of “bound-free” water phases. Dissipative processes of self-organization occur in both living and non-living matter. The same laws govern living and non-living matter, but the mechanism of their manifestation is different. The distinctive feature of living matter is “functional information,” which is an attribute characteristic only of living matter. The ideas of synergetics of self-organization are widespread in modern science. On their basis, the unity of living and non-living nature and the unity of micro- and macrocosms are established—the unity of the two opposites of chaos and order. Functional information is a purposeful indication of the change in the structural-functional state of the living matter. Feedback plays an essential role in self-organization and the dynamic balance of dissipative systems. Self-organization is based on positive feedback (PF). Dynamic equilibrium of the system is ensured by negative feedback (NF). There is a constant competitiveness between the two typ es of feedback - PF and NF. In biological systems, the balance of PF and NF is maintained based on auto-oscillatory homeostasis processes. In the hydration-dehydration processes of biopolymers, the dynamics of the ratio of bound to free water phases coincides with the regulating role of homeostasis. Water sustains the balance of the bound-free water phase ratio, which in turn maintains homeostasis.

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Keywords: Dissipative systems; dissipative self-organization; dissipative regulation; entropy; functional information; system sensitivity; self-oscillations; feedback; homeostasis; bound and free water; hydration, dehydration; biopolymer conformation; information density; synchronization; desynchronization.

1. INTRODUCTION

It is well known that water is the basis of life. Life, as a unique complex form of matter motion, is characterized by self-organization, self-regulation, self-renewal, and self-reproduction of multilevel open dissipative systems [1]. A Dissipative Structure is a thermodynamically open system operating far from thermodynamic equilibrium, that exchanges energy, matter, and information with the external environment. In this kind of systems, organization can emerge through a spontaneous self-organization process, by virtue of the exchanges with the external environment, that generates a formation of both spatial and temporal ordered structures, in which interacting constituents show long-range correlations [1a]. Open dissipative systems are space-time structures that can arise far from thermodynamic equilibrium in a nonlinear region at critical parameter values [2]. Open dissipative systems exchange substance, energy, and information with the external environment [3]. “Dissipative self-organization (synergetic approach) is a process of sorting (spatial, temporal or space-time) in an open system, due to the coordinated interaction of multiple elements that make it up. Hermann Haken gave this definition within the framework of the scientific paradigm, the theory of “joint action” – synergetics [4]. The ideas of synergetics of self-organization are widespread in modern science. On their basis, the unity of living and non-living nature and the unity of micro- and macrocosms are established—the unity of the two opposites of chaos and order.

Matter exists in two forms – matter and field. Matter exists in space and time. Any forms of motion and transformation of matter are impossible without energy. The non-material form of existence and manifestation of the matter is information. Energy and information are integral (attributive) forms of matter [5]. But what exactly is the difference between living and nonliving matter? No chemical elements are the basis of matter peculiar only to living matter in nature. The growth and development of matter also cannot serve as a distinctive and specific feature of a living object. After all, as it is known, crystals grow with the construction and organization of the structure of a particular order. Dissipative processes of self-organization occur in nonliving nature as well. The same laws govern living and non-living matter, but the mechanism of their manifestation is different. In living and non-living matter, information manifests itself differently. It is believed that the distinctive feature of living matter is “functional information [6]. “Functional information” appeared with the emergence of life, as it is associated with functioning complex self-organizing systems, including living organisms. We can also say that “functional information” is an attribute peculiar only to living nature. This is one of the essential attributes separating the living from the non-living objects in nature. In the hierarchy of levels of matter, it is believed that the bridge between living and nonliving matter is supramolecular systems [7].

Supramolecular systems can recognize each other, move, and perform various transformations interacting with other molecules. Various molecular assemblies (genome, organelles, receptors, etc.) with new functional properties characteristic of biological systems can already be formed Fig. 1. And the first functional system of the transitional hierarchical level between non-living and living matter is the cell. Functional information carries targeting structural change. Without targeting, the processes of directed transformation of living matter are impossible. In highly organized matter, intermolecular and intercellular interactions involving functional information form dissipative structures – dissipative systems. Dissipativity is a macroscopic dynamic state of a system (matter) caused by processes occurring at the micro level of this system.

We name some conditions of dissipative self-organization:

1. The system should be open.
2. The system must be in the vicinity of the thermodynamic equilibrium point.
3. The system must contain a sufficient number of interacting elements.
4. Self-organization is based on positive feedback (PF.)
5. Dynamic equilibrium of the system is ensured by negative feedback (NF.)
6. The presence of fluctuations is necessary for the emergence and maintenance of the dynamic order of the system Fig. 2.

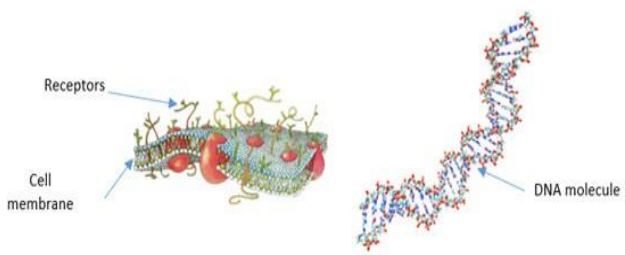

Fig. 1. Examples of supramolecular systems [8]

Fig. 2. Dissipative system – by the example of a flock of starlings (the phenomenon of murmuration) [9]. Similar dissipative structures (systems) are formed within a living organism in the form of an association of molecules and cells of the same type

The cell, as the first functional system, implements one of the fundamental principles of biology – the principle of reduplication (doubling) of hereditary information (matrix principle). The level of living matter usually includes biological organs, organisms, populations, and ecosystems. Biological systems, and their structural and functional organization, are based on the principle of the fractal matrix Fig. 3.

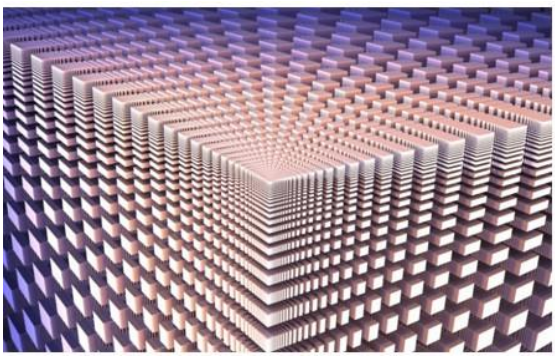

Fig. 3. Example of a geometric fractal matrix. Dissipative systems are dynamic fractal matrices [10]

1.1 What Gives a Living Organism a Large Number of Single- Type Structures Combined into a System?

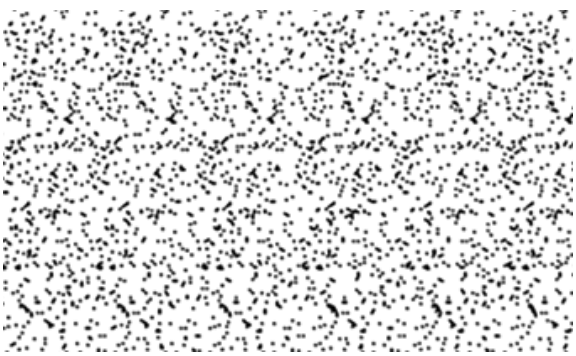

Fig. 4. Static fragment of the dynamics of the relationship of a large number of system elements, demonstrating the statistical regularity (probability) of the predicted behavior of the system

Erwin Schroedinger, an Austrian theoretical physicist and Nobel Prize winner in physics, is one of the developers of quantum mechanics and wave theory of matter. In 1944 Schroedinger wrote his book “What is life in terms of physics?” which significantly influenced biophysics and molecular biology. Schrodinger

showed that “only in the union of a vast number of atoms do statistical laws begin to operate and control the behavior of these unions with a precision that increases with the number of atoms involved. It is in this way that events acquire truly regular features [11].” Behavior of quantum living systems can be described only on the basis of statistical (probabilistic) laws Fig. 4. In essence, dynamic fractal systems (aka dissipative systems) – are a quantum level of matter functioning, with all inherent properties and laws of quantum biophysics. When researching and studying properties of living matter at the level of individual cells and molecules, we often overlook those essential qualities that living matter possesses only in its collective, fractal, dissipative state – quantum state.

1.2 If Life without Water is Impossible, it is Quite Logical to Ask: how does Water Affect the Processes of Self- Organization and Self-Regulation Occurring in Living Matter?

The answer to this question should be sought in biophysical principles of the existence and functioning of living matter based on properties and regularities of operation of dissipative systems. The molecular and cellular level of living matter consists of a vast number of different biopolymers, all processes of the transformation and functioning of which are impossible without the participation of heterogeneous forms of water. Water, a thermodynamic and electrophysical non-equilibrium system, can provide many structure- forming effects and collective quantum processes [12].

Water is a dynamic matrix of dissipative systems of living matter. Regulation processes in living matter, as was mentioned above, take place with the participation of “functional information.” Information in molecular biology makes sense only through the function it encodes – “functional information.” Information is a “differential characteristic” [13]. This means that it occurs when there is some change in the initial state of the structure Fig. 5. Information is contained in the measure of heterogeneity, changes in the conformation of the spatial structure of matter (substance, field), and the measure of system orderliness, connections between individual structural elements of matter. Nature encodes different information in a conformational (structural) way rather than a frequency one. Sources of information inside the organism are large and small molecules. Dynamic changes in molecular structures of living matter are information itself. In this sense, the structure of matter and its information (aka function) in living matter are parts of one whole. Proteins are the most variable structures in the molecular world of living matter. They have the most diverse functional properties. The biological function of a protein is determined not only by its chemical composition but also by its spatial (three-dimensional) structure, which depends, among other things, on the environment in which the protein is located.[14] In the dry state, the protein is electrically neutral and, therefore, inactive. The heterogeneous aqueous matrix in the liquid media of the tissue-extracellular matrix (ECM) and the protoplasmic matrix is active in the auto-oscillatory process of protein hydration-dehydration [14]. This auto-oscillatory process manifests the dynamic charge properties of the protein and changes its

conformation and functional activity. The maximum functional activity of the protein is manifested in the unfolded (hydrated) state [15].

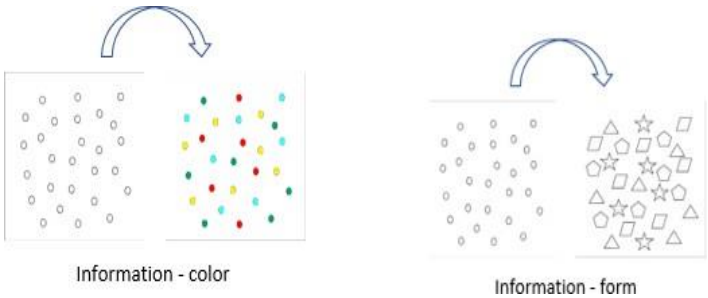

Fig. 5. Example – information as a "differential characteristic

Auto-oscillations are a necessary condition for self-organization in open, dynamic systems to maintain their dynamic stability [16]. Excitation of self-oscillations will always be accompanied by a reduction of the total entropy of the system, i.e., reduction of dynamic chaos and maintenance of the necessary level of order in the system. The auto-oscillatory process in the dissipative system of a living organism provides conditions for maintaining homeostasis. Homeostasis is an integrative dynamic process regulating the functional and morphological (morphofunctional) relative internal constancy of a living organism [17,18]. In homeostasis and metabolic processes, feedback plays an important role. Norbert Wiener, one of the founders of cybernetics and artificial intelligence theory, regarded the feedback principle as the "mystery of life," giving it a fundamental role in the study of living phenomena [19].

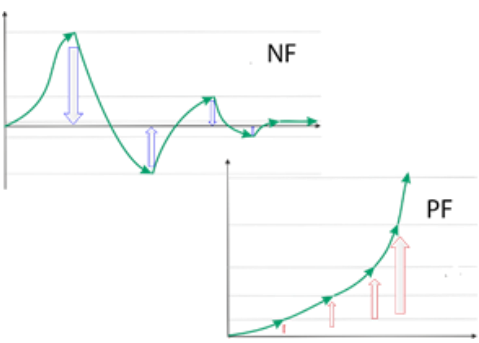

Fig. 6. Directionality of the PF and NF influence on the dissipative system processes

To create new structures and new organs in the process of growth and development of the organism, positive feedback (PF) is necessary, loosening the system (taking this system to a new level of structural organization), and negative

feedback (NF) is necessary to maintain a steady state Fig. 6. The balance of PF and NF ratios ensures the ability of the living organisms both for growth and self-regulation of an individual organism and, as a result, for self-preservation and sustainability of the species. The formation of dissipative structures occurs with the participation of positive feedback (PF) and underlies tissue differentiation during morphogenesis. The preservation of the auto-oscillatory mode in the dissipative structure occurs with the participation of the negative feedback (NF) [20].

Homeostasis can be maintained only under the conditions of the NF, which regulates homeostasis, and the PF, which creates a necessary and sufficient number of interacting system elements and a necessary-sufficient order structure between the elements of this system. Homeostasis is, in fact, an auto-oscillatory process with the NF of information regulation (dissipative regulation), maintaining the constancy of the most important structural characteristics of living matter, the living organism as a whole [21]. Normal homeostasis, as a criterion of health, is related to relative order – the "norm of chaoticity" of structural elements of a dissipative system. For maintaining homeostasis, it is necessary to maintain some "norm of chaoticity" in the regulated system. Both high entropy (excess chaos) and minimal entropy that tends to zero (complete phase coherence) lead to loss of information in living matter and, consequently, to loss of its normal physiological functioning. There is constant competition between two types of feedback – PF and NF. For example, in the nervous system, positive feedback causes enhancement of reaction, which may underlie the development of diseases. Negative feedback, on the contrary, reduces the activity of CNS neurons and response, which is the basis of self-regulation (adaptation). If the balance of feedback is disturbed, PF puts the functioning of the dissipative nervous system into a new functional (morphofunctional) state, which usually leads to pathological homeostasis and even possible destruction (death) of the functioning dissipative system [20].

1.3 What is Water's Role in the Dissipative Regulation of Homeostasis?

Water in the body is represented as free and bound [22]. Free water is a part of the cell cytoplasm, vacuole; it fills the intercellular matrix (ICM), connective tissue. It is necessary for the transport and transfer of substances. Bound water is a part of cellular structures (proteins, membranes) and maintains their structure Figs. 7, 8 [23]. The maximum hydration of biopolymers corresponds to the maximum amount of bound water (gel phase), and dehydration is correlated with an increase in the proportion of free water (sol phase). Note that the concepts of "bound-free" water, "hydration-dehydration," and "gel-sol" states should be perceived as relative, not absolute, bimodal distributions of ratios, these phase aggregate states of dynamic processes (dissipative processes) in living matter Fig. 9. The biphasic state of water is in an auto-oscillatory process of hydration-dehydration of biopolymers. The regulatory (informational) function of homeostasis is carried out in the Gel phase (maximum hydration of biopolymer, maximum amount of bound water). Metabolism, movement of

molecules, tissue growth, development, etc., are performed in the Sol phase. The diffusion rate of substances in the Sol is 10^7 (7 orders of magnitude!) higher than in the Gel [26].

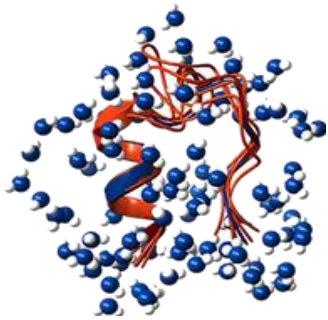

Fig. 7. Hydrate shell of protein molecules [8]

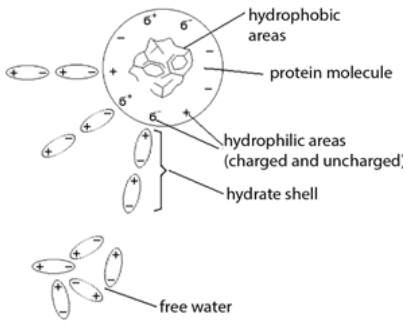

Fig. 8. Free water unbound to biopolymer and bound water as hydrate shell of biopolymer [24]

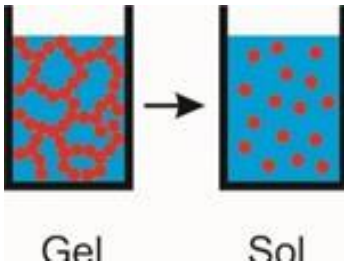

Fig. 9. Example of "gel-sol" states [25]

External energy is consumed during the transition from Gel to Sol, and external energy is released during the transition from Sol to Gel. The rhythms of colloidal

transitions from Gel (gelatinous state) to Sol (liquid state of protoplasm) and back underlie all intracellular movements [26]. External energy is consumed during the transition from Gel to Sol, and external energy is released during the transition from Sol to Gel. The rhythms of colloidal transitions from Gel (gelatinous state) to Sol (liquid state of protoplasm) and back underlie all intracellular movements. Mismatch in the balance of competing processes of NF and PF will manifest itself in changes in the regulatory role of homeostasis and general metabolism, development of diseases, and acceleration of aging. Almost all diseases can be traced to changes occurring at the protein (protein) level [27], with a change in the ratio of water phase states.

The body expends ATP molecules to hydrate protein (create Its maximum unfolded structure). A decrease in ATP in pathologies and aging of the body leads to a low degree of protein hydration, a shift of the Gel phase toward the Sol, a decrease in controlling regulatory processes, and a decrease in overall homeostasis [28]. In this regard, the possibility of homeostasis regulation mediated through the water matrix becomes particularly meaningful. Factors affecting the degree of protein hydration include intrinsic natural (amphoteric) properties of the protein, pH of the environment, and concentration of toxins. The lack of ATP spent on protein hydration, and water structuring can be partially compensated by the intake of negative air ions and water with a negative redox potential (optimally from -50 to -150) [26].

It is important to remember that a decrease in the number of mitochondria and ATP formation occurs in various diseases and in the organism's aging process. Maintaining a high level of homeostasis allows not only to maintain normal metabolism but also to control the growth and development of tissue proliferation between the constantly competing processes of autophagy and apoptosis. At the same time, maintenance of high homeostasis level, and an optimal degree of synchronization (maximum sensitivity) of the dissipative system, allows for achieving maximum information density of regulatory signal with a decreased amount of ATP, i.e., with minimum consumption of the cell's own energy.

Desynchronization of dissipative coupling is necessary for the processes of weakening pathological homeostasis. The information density of the dissipative system can be regulated by an external control parameter coupled with the dissipative system's own field. This control parameter can enhance dissipative coupling by increasing synchronization and weaken dissipative coupling by desynchronizing the system. The cause of many diseases with pathological homeostasis is desynchronization in dissipative systems of normal physiological processes. Disbalance of the psycho- emotional state is correlated with the activity of inflammatory reactions in the body. Inflammatory processes show a correlation with the development of somatic-psychological reactions. At the same time, excessive psycho-emotional states and prolonged stress lead to the development of psychosomatic problems. For example, the excessive response of neurons to the action potential during the stress response is associated with a change in the cell's membrane potential. This, in the structures of the nervous system, causes changes in the excretion of neurotransmitters, neuromodulators,

and neuropeptides, affecting tissue metabolism and homeostasis [29]. Water performs one of the critical functions in maintaining normal homeostasis and metabolism. The organism's adaptation to adverse environmental factors, including psycho-emotional ones, greatly depends on the state of general homeostasis. At the same time, water is the "structural basis of the organism's adaptation" Fig. 10 [28].

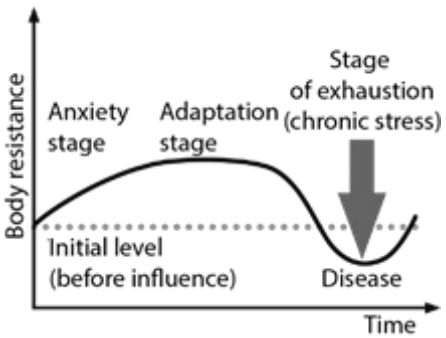

Fig. 10. The body's reaction to stress according to G. Sellier [30]

At the initial stage of stress reaction (stage of mobilization of protective forces), the organism reacts by increasing the proportion of bound water, the maximum proportion of which falls on the stage of adaptation (resistance). With the prolonged persistence of a stress factor for the organism (depletion stage), the phase relation "bound- water" shifts towards an increase in the proportion of free water. In this case, the regulatory function of homeostasis decreases. Risks of the development of pathological conditions increase. Excessive mental reactions, including those caused by drugs that stimulate physical performance, such as Phenamine, reduce the content of bound (structured) water [28]. On the contrary, adaptogens (ginseng, magnolia vine, Eleutherococcus, Rhodiola Rosea, etc.) increase the amount of bound water and decrease the amount of free water. Thus, adaptogens increase the body's nonspecific resistance (increase of adaptation reserves). In acute alcohol intoxication, there is a decrease in bound water and an increase in free water, lasting up to 5 days [28]. The proportion of bound water increases in winter and free water in summer, which also affects homeostasis and metabolism [26,28]. A change in the bound-free water ratio leads to a change in the homeostasis state and the dissipative system's corresponding state. A mathematical function of a strange attractor describes the behavior of a dissipative system. The attractor is a set of trajectories that the system makes in an auto-oscillatory mode Fig. 11 [31].

One of the essential properties of an attractor is its ability to respond to a periodic external influence. This external influence, complementary to the system state, can change the attractor trajectory and switch it to a new form of auto-oscillatory mode and, consequently, to a new homeostasis state. Since the controlling parameter of the dissipative system, functioning by the NF mechanism, is an

external field coupled with the internal field of this dissipative system, such an external field can affect the homeostasis state. The control parameter can enhance dissipative coupling through increasing synchronization and weaken dissipative coupling through desynchronization of the system. Spatially inverse (mirror- symmetric) controls parameters perform desynchronization in the dissipative system. Desynchronization in the dissipative system can be used to reduce the pathological homeostasis function. In addition, a dissipative system is a dynamic multidimensional fractal structure, and the internal field of this structure also has the appropriate volume configuration–- conformation [32,33]. Thus, the external control parameter of a dissipative system (external field) should have a relative complementary conformation and carry a corrective conformation (external functional information), which can switch the attractor of the dissipative system to a new trajectory (vector) of its existence. The properties of molecules and their ability to enter into metabolic reactions and processes are determined by their chemical composition and conformation. Conformation also determines the biological function of proteins [33]. Almost all diseases can be traced back to changes occurring at the protein level [28]. The organism, being a single dissipative system, contains multiple individual dissipative structures (dissipative systems) on different hierarchical levels. Disruptions of homeostasis and metabolism may occur within a single dissipative system and in cross-dissipative links between these systems [34].

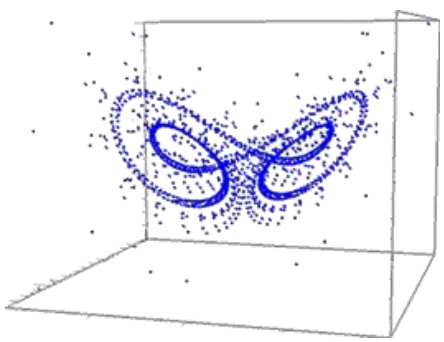

Fig. 11. Example of a dissipative system attractor [8]

2. CONCLUSION

Water, as the basis of life, creates conditions for the emergence and realization of “functional information.” Water provides a dynamic internal environment of all dissipative systems of the organism governed by functional information. Water regulates homeostasis and supports metabolism by maintaining the balance of “bound-free” water phases. Thanks to water, the functional quantum properties of living matter, which create conditions for self-organization, self- regulation, self-renewal and self-reproduction, are manifested in living organisms.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 4
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Exploring Quantum Dynamics of Dissipative Systems in Living Organisms

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ABSTRACT

The functional information of dissipative systems in Living Matter correlates with both the physical and mathematical regularities defining its material and quantum operational levels. The quantum operational level of Living Matter necessitates employing mathematical models to describe spaces of high dimensions. The physical significance of such an approach helps ascertain the meaning of “action information” as the “quantum information” of dissipative systems. Homeostasis is an integrative dynamic process regulating the functional and morphological (morphofunctional) relative internal constancy of a living organism.32 Tissue homeostasis ensures the preservation of the relative stability of the total number of cells, an optimal ratio between dividing cells, differentiated, and apoptotic (undergoing apoptosis) cells within the tissue composition. The competitive balance between a living organism's metabolism and homeostasis is determined by the quantum characteristics of dissipative systems. The characteristics of "quantum information" in dissipative systems show broad patterns that can be used to analyze the mathematical and physical underpinnings of "quantum computers" and materials created using "quantum dots."

Keywords: Dissipative systems; supramolecular systems; functional information; viscoelastic medium; entropy; chaos norm; multidimensional spaces; “action information;” second-order phase transition; wave function; cp symmetry; singularity; “unstable stability;” qubit; quantum dots; self-oscillatory process; positive feedback; negative feedback; metabolism; homeostasis.

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1. INTRODUCTION

The concept of information in the scientific realm has undergone revolutionary changes. Modern methods of information processing in quantum computers, as well as in dissipative systems, are based on the laws and properties of fundamental symmetries [1]. Functional information serves as the organizing foundation of Living Matter. It carries the directive for structural alterations in matter. Directed processes of transforming living matter are impossible without such directives [1a]. Supramolecular systems act as a unifying bridge between “living” and “non- living” matter [2]. It is believed that nature employed supramolecular systems to create biological systems [3].

Supramolecular systems can recognize each other, move, undergo various transformations while interacting with other molecules, leading to the formation of diverse molecular ensembles (genome, organelles, receptors, membranes, etc.) possessing novel collective functional properties characteristic of biological systems. Self- organization, as a process of coordinated interaction, is characteristic of dissipative systems [4]. Dissipative systems are open systems with communicative channels to the external environment for exchanging matter, energy, and information. Structural elements of a dissipative system, existing in collective non-local correlation (“entangled state”), combined within a continuous viscoelastic medium, possess a dynamic order structure.

Our accustomed perception of the surrounding reality is based on the three-dimensionality of space. However, numerous physical properties of Matter cannot be explained, let alone described, based on the principles and laws of three-dimensional space. In this regard, there has been a long-standing quest for possibilities and methods of mathematically and physically describing the properties of matter based on higher-dimensional spaces. Multidimensionality is not an objective reality but a form of perception and interpretation of objective reality by our consciousness, used for mathematical and physical descriptions, often revealing new properties and regularities of studied objects and processes. The dimensionality of space determines the potentially possible number of degrees of freedom, defining the dynamic state and behavior of an object. Contemporary physics considers the “physical reality from the perspectives of geometric, field, and relational worldviews, each of which differs in its own interpretation of the properties of space-time and physical interactions” [5]. Only by considering the specific characteristics of interpreting all three worldviews can a sufficiently comprehensive understanding of physical reality and the role of information in the existing world be established. It is precisely from the standpoint of multidimensionality in space that the possibility arises to describe and more fully reveal the nature of the behavior of various fields—both physical (electromagnetic, gravitational, bosonic, fermionic, etc.) and mathematical (scalar, vector, tensor, etc.). A viewpoint grounded in the multidimensionality of space helps explain the physical properties of “time,” the duality of matter as “wave-particle,” “non-localities,” and “information” [6].

The first concept of the world's multidimensionality, four- dimensionality, emerged in the works of H. Minkowski as an additional coordinate to the three dimensions. This fourth coordinate's physical significance is attributed to the concept of "time" and its physical properties. The property of time in the four-dimensional space possesses the directionality of the curvature gradient in space. Depending on the direction and degree of the gradient, it can influence matter and the processes occurring with it. Processes influenced by the fourth coordinate-time can either accelerate or decelerate. Subsequent development in the mathematics of curved surfaces by N. Lobachevsky and B. Riemann allowed for describing and systematizing phenomena in the micro- and macro-worlds based on the properties of five-dimensional spaces. The physical significance of such descriptions defines a new physical reality (a new parameter) of the five-dimensional space as the "action coordinate." The action coordinate of the five-dimensional space, when interacting with a physical object, can convey action information to this object about the directionality of changing its dynamic properties. Initially, the physical significance of the fifth coordinate was considered in the micro-world scales of fundamental particles as a discrete coefficient of energy comparable to Planck's constant.

This fundamental physical constant - Planck's constant, as a unit quantum of action or quantum of quantization energy, can be considered the primary quantum of action information. Simultaneously, the fifth topological coordinate - the "action coordinate" in the macro manifestation of dissipative systems defines its interaction with an object in the five-dimensional space (dissipative system) and represents a variant of the fermionic field of this space. From a mathematical perspective, action information is a derivative of the moments of impulses of elements constituting the dissipative system and essentially represents the dispersion of the density distribution of these moments of impulses, constituting the density of action information that determines the evolution and behavior of the dissipative system. In this regard, the "fermionic field," as the multi-vector dynamics of the distribution of "action information" density, is sometimes referred to as a "spinor field." However, it's essential to note certain distinctions: the "spinor field" can be either fermionic or bosonic [7]. This is explained by the fact that spin can be both integral and fractional. Fermions possess fractional spin, while bosons have integral spin. The density of "action information" of dissipative systems emerges during a second-order phase transition and is linked to symmetry breaking (the appearance of anisotropy within the system) and the emergence of high susceptibility (sensitivity) of the dissipative system to weak internal and external regulatory signals. During a second-order phase transition, elements of the dissipative system are in states close to a quantum superposition, i.e., in a non-local correlation state.

According to the theory of "mean field" by Nobel laureate physicist Academician Lev Landau, abrupt second-order phase transitions lead the dynamics of the dissipative system into a commensurable and coordinated state with the field it generates [8]. Consequently, the dissipative system becomes controllable by an external field. Therefore, an external field could become the controlling parameter capable of adjusting the state [9]. Another important effect of Landau's

"mean field" theory is the deduction that a dissipative system undergoing a second- order phase transition transitions its elements' interaction from short-range to long-range, becoming non-local [10,11]. Quantum states and processes manifest randomness and can only be described based on statistical (probabilistic) mathematical characteristics. One of these characteristics is the wave function, introduced by Erwin Schrödinger- an Austrian theoretical physicist, Nobel laureate in physics, and one of the developers of quantum mechanics and the wave theory of matter [12]. The wave function determines the nature and dynamics of the probabilistic behavior of a quantum particle or system. Properties of quantum information are described by wave functions of fermionic and bosonic fields in multidimensional spaces. For living matter, static states are impossible [13]. Any processes within living matter, combined into dissipative systems, exist in a self-oscillatory regime and, from a mathematical standpoint, are a strange attractor, which in Riemann's five-dimensional space transforms into a "torus." It is precisely within the mathematical model of a torus that the non-locality properties of dissipative systems are well demonstrated [14].

In an absolute quantum state, matter is unmanifested but possesses maximum information density (quantum information) and exists in a state of maximum quantum coherence. Quantum information ensures an inseparable connection between the material and field states of matter. The transition from an absolute quantum state to a manifested material state occurs at the moment of wave function collapse. Despite dissipating being associated with a form of decoherence, a significant achievement in modern quantum physics has been the discovery of the opposite, constructive role of dissipation in generating entangled states [15]. Different interpretations exist regarding the peculiarities of the meaning of the wave function. Professor of Applied Mathematics at Växjö University, Sweden, and Director of the International Center for Mathematical Modeling in Physics and Cognitive Sciences A. Yu. Khrennikov notes that one physical interpretation of the wave function is the "density of interference of probabilities." Quite recently, interference of probabilities was obtained within classical probability theory, showing the importance of the quantum system's interaction with various conditions of the external environment [16].

In 1927, Niels Bohr proposed using the "Complementarity Principle" to describe the properties of matter, stating that for a more complete description of matter, both its wave and particle characteristics should be considered. Essentially, Bohr's "Complementarity Principle" defines Matter as "Substance and Field," considering their quantum properties [17].

One of the widely known fundamental particles with wave properties is the photon. The photon, as a quantum of the electromagnetic field (specifically, a quantum of light), acts as a carrier of electromagnetic interaction and information. The relationship between the field and charge is defined by Maxwell's equations in classical electromagnetic theory. Hence, fundamental laws of symmetry (laws of invariance), which define the physical properties and mathematical interconnection of basic physical quantities - charge, energy, momentum, angular momentum - with space and time, can be transposed to operations involving

electromagnetic waves (photons). One such law, CP symmetry (combined parity law), reflects the dynamic complex-conjugate interaction of numerous phenomena functioning within matter at different hierarchical levels. The CP symmetry law corresponds simultaneously to charge and spatial inversion. The CP symmetry indicates a generalized principle of conjugating the "material" and "quantum" within living Matter, as a necessary condition for its dynamic balance in a state of "unstable stability," corresponding to the principle of existence of living dissipative systems. The formation and storage of biological information, functional information, are fully related to the CP symmetry law. In this context, the principle of "mathematical singularity" of CP symmetry relates to the concept of "biological singularity" [18].

Singularity is a special point at which singleness of existence, events, or phenomena can simultaneously manifest or vanish. Mathematical singularity (peculiarity) is a point where a mathematical function tends toward infinity or exhibits some other irregular behavior. Biological singularity is connected to the biological evolution and mathematical singularity of living Matter [19]. Ancient philosophers in their intuitive models unified the principle of "unstable stability" around the point of singularity, correlating it with the concept and properties of the "Great Limit," where in singleness of existence, events, or phenomena arises (Fig. 1). Based on these properties of CP symmetry, Academician Lev Landau proposed in 1957 that CP symmetry should be considered as the true symmetry between matter and antimatter, capable of leading to particle annihilation.9 The structural-functional manifestation of the "Great Limit" in conjunction with the fundamental laws of symmetry can be demonstrated through a model of a cube where complex-conjugate functions of various spatial-functional (structural-functional) interactions are located at its vertices. The intersection of the inner diagonals of the cube spatially defines the "Great Limit" as the boundary transition between material and quantum states and illustrates the principle of superposition between these states. In Fig. 2, marked with the number "1." The unitary essence of the "Great Limit" arises from the mathematical operation "composition":

$$e^{ibx} \circ e^{-ibx} = 1$$

This formula combines the interaction of complex-conjugate functions, which can be interpreted as spirals with opposite twisting directions (Fig. 1). From a physical standpoint, such a model corresponds to the interaction of opposite charges or electromagnetic waves with right and left polarizations. Displacement of the function from the "Great Limit" leads to symmetry breaking, the emergence of imbalance, and anisotropy in the environment. The distinguished scientist V.I. Vernadsky stated that "Only the non-equilibrium state of 'leftness' and 'rightness' in the biosphere ensured the emergence of life on planet Earth" [20]. Another renowned scientist, the French physicist Pierre Curie, noted that "dissymmetry generates action; if there is no dissymmetry, then the phenomenon is impossible" [21]. Quite recently, the scientific team of astronomers from the Sloan Digital Sky Survey (SDSS) under the leadership of Michael Longo (USA) demonstrated through years of research that the current state of the Universe is both non-

equilibrium and integratively right-handed [22]. Consequently, it is evident that life on Earth integratively evolved as right-handed.

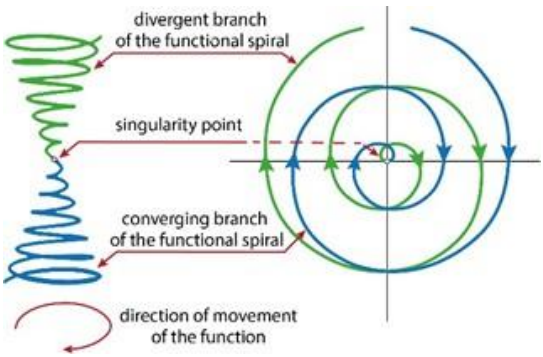

Fig. 1. Dynamic state of the "Great Limit" as a model of "unstable stability" around the "point of singularity"

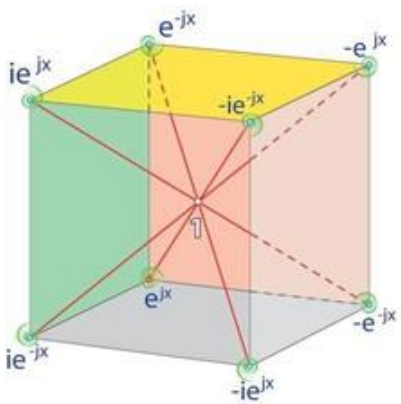

Fig. 2. Model of a cube with complex-conjugate functions at its vertices.

Living matter is known to possess relative chiral purity in its constituting biopolymers. Proteins and their derivatives predominantly contain "left-handed" amino acids (L-amino acids), while nucleic acids (DNA and RNA) contain only "right-handed" sugars (D-sugars). The polarization of our cell's cytoskeleton is right-handed, and the cumulative polarization of the whole organism is also right-handed [23].

The functioning basis of Dissipative systems in living organisms is also right-handed asymmetry. One of the fundamental fields, the electromagnetic field, governs interacting processes in living matter. Hence, many physiological processes may be regulated by flows of electromagnetic radiation of right

polarization. Prolonged exposure to left-polarized flows may lead to changes or even disruption of natural physiological processes. In 2012, at the Nizhny Novgorod State Medical Academy in the laboratory of the Department of Physiology, research was conducted on the proliferative capacity (cell division) of human liver cell line cultures of both normal and neoplastic lines and rat glial cells under the influence of fields of right and left polarization. The study results showed that the field of right polarization significantly suppressed the metabolism and proliferation of neoplastic cell lines [24]. The field of left polarization activated metabolism and accelerated the proliferation of neoplastic cell lines. Conversely, the field of left polarization slightly reduced the activity of metabolism and proliferation of normal cell lines. On the contrary, the field of right polarization gently stimulated the metabolism and proliferation of normal cell lines [25].

The concept of information in the scientific realm has undergone revolutionary changes. Modern methods of information processing in quantum computers, as well as in dissipative systems, are based on the laws and properties of fundamental symmetries. The fundamental notion in classical information theory is the “bit,” which takes values of 0 or 1. The paradigm shift in our understanding of information began with replacing the classical “bit” with its quantum analog - the “qubit.” Photons, serving as the foundation of qubits in quantum computers, carry information about the direction of its propagation (Poynting vector) and polarization (axial vector) (Fig. 3) [26]. A qubit comprises 8 cubits. Cubits within the qubit can exist in an equilibrium (metastable) state, where information is absent, analogous to the state of the “Great Limit.” Altering the equilibrium state of the cubits results in the emergence of information, described in the qubit by a vector. This vector is the outcome of the interaction between polar and axial vectors, describing the direction of information propagation and polarization in space. Any non-equilibrium state of the qubit contains “action information.” Quantum computers essentially operate with the properties of information in a five-dimensional space, possessing additional degrees of freedom. The information density of a qubit significantly surpasses that of a bit. Photons in quantum computers, existing in a state of superposition (quantum non-locality), represent a quantum dissipative system. In 2022, the Nobel Prize in Physics was awarded to scientists Alain Aspect (Palaiseau, France), John F. Clauser (California, USA), and Anton Zeilinger (Austria) for experiments with entangled photons, establishing the violation of Bell inequalities, and for their contributions to quantum informatics (Fig. 4) [27].

The described qubit model by mathematicians and programmers is transposed into its simplified model, the “Bloch Sphere,” which considers the states of polar and axial vectors, defining the wave function ψ of the qubit. It’s worth noting that it’s impossible to fixate any intermediate position of the wave function due to quantum laws: any measurements will only yield 0 or 1, and determining the relationship of complex coefficients will only be possible through numerous repetitions and probability calculations. Another equally interesting example of quantum dissipative systems is “quantum dots.” In 2023, the Nobel Prize in Chemistry was awarded to scientists Mungi Bawendi (Massachusetts Institute of Technology), Louis Brus (Columbia University), and Alexei Ekimov (Nanocrystals

Technology Inc) from the USA for the development and synthesis of “quantum dots.” Devices utilizing materials with “quantum dot” properties allow obtaining emissions across a vast range of discrete pure colors within the optical spectrum [28]. Despite the Nobel Prize being awarded in the field of chemistry for the development and synthesis of materials with “quantum dot” properties, the “quantum dots” themselves, as objects of study, are closely related to physics. The physical principle of an optoelectronic crystal, consisting of “primary cells” - “quantum dots,” can be associated with the complex-conjugate functions of the “Great Limit,” described earlier, which, on an energetic level, enables the control of the crystal’s operation.

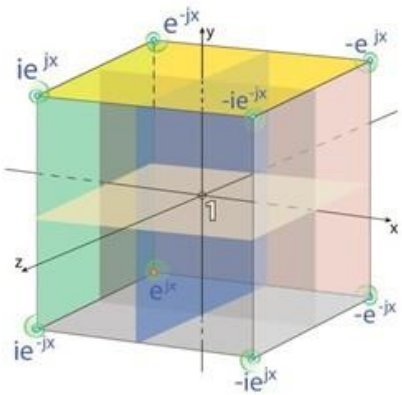

Fig. 3. Model of a qubit with embedded cubits

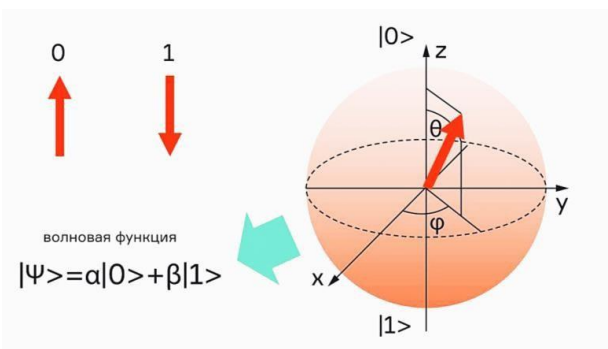

Fig. 4. “Bloch Sphere” as a qubit model

Systems exhibiting “unstable stability” properties can be associated with dissipative systems functioning far from the point of thermodynamic equilibrium. The state of functional systems under “unstable stability” from a mathematical perspective can be characterized as an auto-oscillatory process with trajectories

of a strange attractor or a torus. An essential characteristic of auto- oscillatory processes is the presence of negative feedback (NFB) [29].

Quantum dots, as a particular state of the optoelectronic state of matter, are characterized by the fact that their operation is only possible under a non-equilibrium initial state of the valence layer of semiconductor crystal atoms, which can be correlated with the complex-conjugate functions of the "Great Limit," ensuring high sensitivity to external control signals, leading to emission within a narrow, specific range. It's important to note that the existence of "quantum dots" of matter (where the quantum-material state of matter is realized) might not be possible in all types of materials. They can be obtained under one crucial condition: the "primary cell" forming the quantum dot must possess one critical property: the atoms constituting the material of this primary cell should form a complex-conjugate structure, the generalized valence band of which contains 8 electron clouds, corresponding to the maximal filling of the energy levels of this cell. It is precisely this combination of chemical elements of the "quantum dot" that ensures a metastable (unstable stability) state of electron clouds, capable of reacting to an external control signal and transitioning to a higher energy level of the "conduction band," surpassing the "forbidden zone" [30].

At the moment of the electron's return to the initial state in the "valence band," narrow-band radiation of monochromatic light quanta occurs. An electron that transitions to the conduction band retains a superposition state with electron clouds of the "valence band" of the quantum dot. A single cell of a quantum dot containing 8 electron clouds can be compared to the property of the "Great Limit" cube, where the equilibrium state of the cube's diagonals at point "1" represents the true "Great Limit" formed by the equilibrium state of the 8 cubits of this cube. The existence of 8 interaction combinations is a systemic condition for building and stable functioning of non- equilibrium systems both in inanimate and living Matter. Such a state allows, with the presence of a correctly selected external control parameter (factor), the control (regulation) of processes in Matter, maintaining it in this non-equilibrium auto-oscillatory state according to the NFB principle. Fluctuations and Self-Oscillations via NFB as a Necessary Condition for Self-Organization in Open Dynamic Systems to Maintain their Dynamic Stability. The excitation of self-oscillations (the emergence of auto generation) will always be accompanied by a decrease in the total entropy of the system, i.e., a reduction in dynamic chaos and the maintenance of the necessary level of order in the system. An auto-oscillatory process in the dissipative system of a living organism provides conditions for maintaining homeostasis [31].

Homeostasis is an integrative dynamic process regulating the functional and morphological (morphofunctional) relative internal constancy of a living organism [32]. Tissue homeostasis ensures the preservation of the relative stability of the total number of cells, an optimal ratio between dividing cells, differentiated, and apoptotic (undergoing apoptosis) cells within the tissue composition. In the processes of homeostasis and metabolism, feedback loops play a significant role. Norbert Wiener, one of the founders of cybernetics and artificial intelligence theory, considered the feedback principle as the "mystery of life," assigning it a

fundamental role in studying phenomena in the living nature [33]. For the creation of new structures, new organs in the process of organism growth and development, positive feedback (PFB) is necessary, destabilizing the system (bringing this system to a new level of structural organization). For maintaining a stable state, negative feedback (NFB) is necessary [34].

The balance of PFB and NFB relationships provides the ability of living organisms both to grow and self-regulate individual organisms and, ultimately, self-preservation and species stability. The formation of dissipative structures involves positive feedback (PFB) and forms the basis of tissue differentiation in morphogenesis. The preservation of the auto-oscillatory regime in a dissipative structure occurs with the involvement of negative feedback (NFB) [35].

Maintenance of homeostasis is only possible with NFB, which actually regulates homeostasis, and PFB, which creates the necessary and sufficient number of interacting elements of the system and the necessary-sufficient structure of order between these system elements. Homeostasis, essentially, is an auto-oscillatory process with NFB of informational regulation (dissipative regulation), maintaining the constancy of vital structural characteristics of living matter, the entire living organism [35]. Normal homeostasis, as a health criterion, is related to relative order - the “norm of chaos” of structural elements of the dissipative system [36]. Both high entropy (excessive chaos) and minimal entropy striving towards zero (complete phase coherence) lead to the loss of functional information in living matter and, consequently, the loss of its normal physiological functioning [37]. The value of the “norm of chaos” (entropy norm) is a systemic criterion of human “health norm.” There is a constant competition between two types of feedback - PFB and NFB. The spatial-temporal dynamics of a dissipative system’s state, associated with its degree of chaos, is the result of the competition between these two types of feedback. Positive feedback (PFB) determines the growth, formation, and emergence of a dissipative system by ensuring a sufficient and necessary number of elements of this system. The formed dissipative system possesses several energetic characteristics: temperature, pressure, Gibbs energy. However, for example, in the nervous system, excessive positive feedback causes an increase in the reaction, which may underlie the development of diseases. Negative feedback, on the contrary, reduces the activity of CNS neurons and the response, which underlies self- regulation (adaptation). If the balance of feedback is disturbed, the PIC brings the functioning of the dissipative nervous system into a new functional (morphofunctional) state, which usually leads to pathological homeostasis and even possible destruction (death) of the functioning dissipative system [38].

The formed new state of the system occurs simultaneously with the involvement of all its constituent components [39]. Such a new state of the system carries a new regulatory (informational) signal, complementarily significant already for other levels of cascade interaction and regulation. Moreover, the dissipative nature of the regulatory signal is formed and uniformly—essentially non-locally—perceived by all elements of the dissipative system with minimal external influences on it. At the same time, the viscoelastic medium of the dissipative

system performs the function of a “receiver,” a converter capable of maintaining or changing the informational density of the regulatory signal, and an “amplifier” [40]. The properties of viscoelastic interaction between the elements of the dissipative system through its viscoelastic medium provide a non-local, essentially quantum character of Living Matter’s behavior.

2. CONCLUSION

The dissipative systems of a living organism are the connecting link between the material and quantum states of the processes occurring in Living Matter. The quantum properties of dissipative systems of living matter indicate the need to perceive the multidimensionality of the space of the surrounding world, which opens to our understanding its special physical properties.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 5
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Comprehensive Assessment of the Taxonomic and Phylogenetic Status of the Celeastralean Plexus

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ABSTRACT

The taxonomic and phylogenetic status of the *celeastralean* plexus is thoroughly evaluated and reported. A synthetic review has been attempted using the data from several disciplines disclosed by previous writers as well as the current author's alliance study. The taxonomic literature indicated that the *Celeastrales* (*sensu lato*) are a loose-knit assemblage. The tribal, subfamilial, familial and even ordinal boundaries are uncertain and even criss-cross each other. It appeared that the alliance can be grouped under two taxonomic entities viz., the *Celastrales* and the *Rhamnales* which appear evolved convergently.

Keywords: Taxonomy; phylogeny; celastrales.

1. INTRODUCTION

The order *Celastrales (sensu lato)* is a loose - knit assemblage. The taxonomic history clearly reflected that this alliance is not restricted to any taxonomic entity. c. It is unclear where different taxonomic entities end and where they begin based on exomorphic traits. The partnership has been fairly examined in a variety of endomorphological contexts. However, the data so accrued is not assessed critically to date. If at all assessed, they are assessed using information from a single discipline. The present authors studied vegetative anatomy of some *Celastraceae, Hippocrateaceae and Rhamnaceae.* They are also borrowed evidence from past literature and reviewed synthetically. The result of our in - depth study in this alliance are being presented in this communication.

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2. TAXONOMIC HISTORY

In the treatment of Bentham and Hooker [7], the genera of the family *Celastraceae* and *Hipporcrateaceae* constitute a composite family, the *Celastraceae (sensu lato).* They categorised these genera into two tribes - *Celastreae* and *Hippocrateae.* The tribe *Hipporcrateae* included only four genera *viz*., *Hippocratea, Salacia, Siphonodon and Llavea*, whereas the others are kept under the tribe Celastreae. The family *Celastraceae* (sensu lato), in their scheme, is kept under the order Celastrales alongwith the families such as *Rhamnaceae, Vitaceae and Stackhousiaceae*. The family *Rhamnaceae* is divided into four tribes *viz*., *Ventilagineae, Zizypheae, Rhamneae* and *Gouanieae.* Bessey [9] included the families *Rhamnaceae, Vitaceae, Celastraceae* and an independent family *Hipporcrateaceae* in the same order *Celastrales* alongwith some others.

In the Englerian treatment, Engler and Diels [17], the three families-Celastraceae, Hipporcrateaceae and Rhamnaceae are treated under two independent orders viz., the Sapindales and Rhamnales. The former two families are included in the Sapindales alongwith other 22 families. The Hipporcrateaceae are accorded an independent familial status. The family Rhamnaceae is included under the Rhamnales alongwith the Vitaceae only. In the latest Engler's syllabus, Melchior [51] included the Celastraceae and Hipporcrateaceae as two independent families within his order Celastrales alongwith other 11 families. The family Celastraceae in his system is divided into five subfamilies *viz.*, Celastroideae, Tripterygioideae, Cassinoideae, Goupioideae and Siphonodontoideae. The families have been divided again into different tribes. However, he is silent about further categorization of his family Hippocrateaceae. His order Rhamnales contains the families Rhamnaceae, Vitaceae and Leeaceae. The family Rhamnaceae has been divided into five tribes *viz.*, Rhamneae, Zizypheae, Ventilagineae, Colletieae and Gouanineae. Treatment by Wettstein [100] is essentially similar in regard to the placement of the three families-the Celastraceae, Hipporcrateaceae and Rhamnaceae. Only Melchior [51] included Leeaceae under the family Vitaceae and accorded to it a subfamilial status.

Rendle [69] included the family Celastraceae in the order Celastrales, alongwith Staphyleaceae, Aquifoliaceae and Empetraceae, whereas the family Rhamnaceae is kept under the order Rhamnales alongwith the family Vitaceae. He included the Hipporcrateaceae *(sensu stricto)* under the Celastraceae (*sensu lato*) the Leeaceae under the Vitaceae. Benson's [6] order Sapindales contains the Celastraceae and Hipporcrateaceae as separate families alongwith many others, few of which he considered of uncertain position. He also included the families Rhamnaceae and Vitaceae (*sensu lato*) under his order Rhamnales.

Hutchinson [31] in his revised system placed the Celastraceae and Hipporcrateaceae as distinct families alongwith few others under the order Celastrales, whereas his Rhamnales contained the families *viz.*, Rhamnaceae and Vitaceae alongwith Heteropyxidaceae and Elaeagnaceae. Takhtajan, [94,95]

recognised the Celastraceae and Hipporcrateaceae as independent families and included them under his Celastrales alongwith some others; few of which, in his opinion, are of doubtful position. He includes the Rhamnaceae and Vitaceae under a single order Rhamnales; however, the latter has been divided into two separate families- the Vitaceae and Leeaceae. Cronquist [10,11,12] recognised the order Celastrales as containing the separate families Celastraceae and Hipporcrateaceae alongwith few others. His order Rhamnales includes the families Rhamnaceae and Vitaceae; the Leeaceae has been given a separate familial status.

Soo [87] in his review of the new classification systems of flowering plants included only two families within the Celastrales-the Celastraceae and Aquifoliaceae. Likewise, his order Rhamnales contains the Rhamnaceae and Vitaceae only. He appears to have included Hipporcrateaceae under the Celastraceae (*sensu lato*) and Leeaceae under the Vitaceae (*sensu lato*).

Thorne [96] in his recent treatment included Celastraceae under his order Celastrales but divided it into five subfamilies *viz*., Celastroideae, Tripterygioideae, Cassinoideae, Hippocrateoideae and Siphonodontoideae. The genera Goupia and Lophopyxis are kept under their independent families - the Goupiaceae and Lophopyxidaceae respectively. Hipporcrateaceae (*sensu stricto*) is accorded a subfamilial rank under the family Celastraceae (*sensu lato*). He included the family Rhamnaceae and Elaeagnaceae in his order Rhamnales. It is interesting to note that the family Vitaceae has been kept under the order Cornales. The family Vitaceae has been divided into two subfamilies *viz*., Vitoideae and Leeoideae.

3. EXOMORPHOLOGICAL SIGNIFICANCE AND TAXONOMY

Some systematists (Bentham and Hooker,[7]; Rendle, [69]; Soo, [87]; Thorne, [96], submerged the genera of Hipporcrateaceae into the Celastraceae (*sensu lato*), while others (Engler and Diels, [17]; Melchior, [51]; Westteein, [100]; Benson, [6]; Hutchinson, [31.32,33]; Takhtajan, [94,95]; Cronquist, [10,11,12], and split the latter into the Celastraceae proper and the Hipporcrateaceae. Engler and Diels [17] distinguish the family Celastraceae on the basis of two ovules per locule and seeds arillate from the family Hipporcrateaceae exhibiting predominant lianas habit, usually opposite leaves and winged angular seeds. However, Melchior [51] delimits these two families on the basis of stamen number and type of seeds, stamens 4-5 (rarely 10) and seeds arillate in the Celastraceae, while stamens are mostly three in the Hippocrateaceae. Benson [6] emphasizes other characters- anthers introrse in the Celastraceae and extrorse in the Hippocrateaceae.

Hutchinson [31] also laid emphasis on number of stamens (usually four to five) and nature of seeds, and the seeds with copious endosperm in Celastraceae, whereas stamens only three and seeds without endosperm in Hippocrateaceae. Cronquist [1112] employed the number and position of stamens, presence or absence of endosperm, aril and latex- system to circumscribe these two families. Bentham and Hooker [7] although include the Hipporcrateaceae into the Celastraceae, they divide the latter into two tribes *viz.*, the tribe Celastreae and

the tribe Hippocrateaceae based on stamen number and albuminous or exalbuminous seeds. This clearly shows that these authors are not consistent in regard to the taxonomic criteria to be used to delimit the families Celastraceae and Hippocrateaceae.

Engler and Diels [17] kept the families Celastraceae, Hippocrateaceae and few others under their order Sapindales and distinguished from the order Geraniales in the ovules pendulous with the dorsal raphe and micropyle upward or erect with the ventral raphe and micropyle downward. They entreated the families Rhamnaceae and Vitaceae in the order Rhamnales and distinguished it from the order Geraniales and Sapindales by the stamens in a single whorl, as many as sepals and opposite the petals, the ovary usually with one or two ascending ovules. Benson [6] employed the same features of staminal position in the flower of this alliance. The features which have been exploited at ordinal level also do not appear uniform for this alliance in the different systems of classification. Engler and Diels [17] stressed simple leaves, perigynous flowers, antipetalous stamens and basal ovules in the Rhamnaceae to distinguish it from the family Vitaceae which show predominantly climbing habit, presence of tendril, antipetalous stamens, axile placentation and berried fruit. Benson [6] exploits the characteristics of sepals, fruits and presence of tendril to delimit these families. Rendle [69] characterizes the order Celastrales by flowers bisexual or unisexual by abortion, regular hypogynous cyclic, four to five numerous petals free or sometimes connate at base, stamens alternate to petals and few other ovarian and ovular features. He distinguishes the order Rhamnales from the Celastrales giving emphasis on antipetalous stamens, the other features are overlapping in the Celastrales. Melchior [51] and Wettstein [100] emphasized similarity. Hutchinson [31,34] stressed more on position of nectariferous disc in the flowers, position of stamens, aestivation of petals, presence or absence of endosperm and nature of leaves while defining these orders. Hutchinson [31] used pellucid, punctate leaves, inflorescence leaf opposed, presence of tendril and fruit baccate in Vitaceae, whereas these are not so in the Rhamnaceae. Cronquist [11] in his synthetic assessment of this alliance used large number of exomorphic as well as endomorphic features. Takhtajan [94,95] also distinguishes the order Rhamnales by the antitepalous stamens from that of the Celastrales wherein the stamens are alternate to petals. Bentham and Hooker [7] placed the families Celastraceae and Rhamnaceae under the same order Celastrales and differentiated on the basis of stamens alternate to petals in the Celastraceae and opposite petals in the Rhamnaceae. These families also find place in different orders, the Celastraceae in the order Celastrales and the Rhamnaceae under the order Rhamnales. Cronquist [10,11,12] employed the position of stamens in relation to the petals for the delimitation of the orders Celastrales and Rhamnales. They are similarly treated by Takhtajan [94,95].

4. SYNTHETIC ASSESSMENT OF CELASTRACEAE AND RHAMNACEAE

The above resume of systematic treatment of the families Celastraceae (sensu stricto), Hipporcrateaceae *(sensu stricto)* and Rhamnaceae reveals that there has been no unanimity of opinions in earlier and even in the current systems of

classification. They reveal considerable diversity of opinions in regard to their position, familial circumscriptions and association with other families in a group. There are certain criteria which distinguish and delimit certain subgroups, while there are others that hold them together into larger groups.

5. VEGETATIVE ANATOMICAL EVIDENCE

While epidermal and other vegetative anatomical evidence are of utility in the appraisal and evaluations, they have their own limitations as an effective tool in interpreting the putative phylogenetic assessments and taxonomic delineations. The present authors, in addition to their own observations, employed data from studies of earlier authors for a more comprehensive discussion of the problem. In the following paragraph is attempted an assessment of the Celastroids and Rhamnoids to focus the intricacies of the situation. Evidence from other domains of plant morphology is freely borrowed in this assessment. Based on this resume, conclusions, as reasonably as can be arrived at, are drawn.

Majority of plants of the three families exhibit three- lacunar, three-traced nodes (Shisode and Patil [71,74] few taxa, however, show unilacunar one-traced nodes, the intermediate two-lacunar, two-traced condition is found in *Ziziphus caracutta, Z. mauritiana* and *Z. nummularia* of the Rhamnaceae. The three-lacunar, three-traced nodes are basic. The unilacunar, one-traced is derived one in all the three families investigated. Thus anatomy of nodes represents evidence more to indicate similar development and evolutionary trend in these three families.

The present author's investigation (Shisode and Patil [73,76,71,76] and those summarized by Metcalfe and Chalk [5] show anomocytic and anisocytic types more common in the three families. Other types such as paracytic, diacytic, tricytic, cyclocytic and co-pericytic are found sporadically in the taxa studied. Hartog and Bass [29] made more or less similar observations in the members of Celastraceae. It is interesting to note that hexacytic stomata occur exclusively in *Colletia cruciata* for which it is of diagnostic value. Likewise, paracytic type is noted for Kurrimia. The present study shows that majority of species in the three families have hypostomatic leaves; very few are amphistomatic. Pant and Kidwai [65] and Metcalfe and Chalk [54] also noted hypostomatic condition of the leaves in the Celastraceae. Similar condition is also noted in Hipporcrateaceae by Metcalfe and Chalk [54]. This may be a significant ecological parameter but it is of little phyletic value. Tannins are common in most of the plants in the three families investigated (Shisode and Patil [73,76,71,80].

Clustered crystals are common in the petiole of Rhamnaceae. They are totally absent in the Hippocrateaceae and present only in *Eunonymus fortunei* of the Celastraceae. (Shisode and Patil, [73] They are, however, occasionally present in the stem-axis. However, they are exclusively present in the leaves of Gouania microcrapa of the Rhamnaceae. Metcalfe and Chalk [54] consider the occurrence and distribution of crystals of specific rather than generic diagnostic value. It is interesting to note that raphides occur only in the leaves of *Euonymus fortunei* of the Celastraceae. The occurrence of clustered crystals and raphides are of

diagnostic value but appear to be of little phylogenetic significance. Anatomy of the petiole, stem and leaves in the three families presently studied reveals an interesting fact that secretory cavities are present only in the species of Rhamnaceae (Shisode and Patil [78,81]. They are totally wanting in the Celastraceae and Hippocrateaceae (Shisode and Patil, [75].

Metcalfe and Chalk [54] summarized and reviewed different anatomical characters of the three families presently investigated and indicated the general similarity of the anatomical characters of Hippocrateaceae to those of majority of the Celastraceae. They further stated that these two families are closely related to one another. They also supported close relationship on the basis of exomorphic features of these two families.

Metcalfe and Chalk *(loc.cit.)* drew attention to the inclusion of some genera like Kurrimia, Perrottetia and Tripterygium in the Celastraceae. In their opinion, these genera differ considerably from one another and also from the remainder of the Celastraceae. In their opinions, these genera differ considerably from one another and also from the remainder of the Celastreceae. They considered the genus Goupia definitely aberrant within the Celastraceae and erected an independent family Goupiaceae (Shisode and Patil, [82] Metcalfe and Chalk [54] pointed out to the occurrence of grouping of the species of *Ziziphus* on wood anatomical features; one is characterized *by* diffuse apotracheal parenchyma as in *Ziziphus angolita, Z.sonorensis, Z.mistol* and *Z.spinachristi.* Likewise, they also recognised grouping of species within the genus Rhamnus based on wood anatomical features.

The outer surface of epidermis is smooth or may show cuticular ornamentation in the form of papillae or striations. (Shisode and Patil [73]. The development *of* epidermal papillae is notable in *Maytenus ovata* and *M.rothiana* of the Celastraceae. They are formed on the adaxial surface of leaf in case of *Maytenus ovata,* whereas on the abaxial in case of *Maytenus rothiana (Shisode and Patil, [73]* They have been also noticed in case of *Celastrus paniculatus* and *C.stylosus* on the midrib and veinlets of the abaxial foliar epidermis by Pant and Kidwai [65] The cuticular striations on the foliar epidermis are wanting in the Celastraceae and Hippocrateaceae. Exceptionally, they are present on mid-vein cells of *Reissantia grahamii.* They are observed in fairly good number of plants of the family Rhamnaceae (Shisode and Patil [76] They are observed on the upper foliar surface, mid-vein cells and the cells of veinlets *Pomaderris apetala* and *Rhamnus wightii.* They are noticed only on the upper foliar surface but not on the veins and veinlets in case of *Scutia rnyrtina.* In *Colubrina asiatica, Ziziphus nummularia* and *Z.rugosa* they are present on the lower foliar surface of the cells of midvein and veinlets. The occurrence of cuticular striations appears to be of systematic value. These are also thought so in other taxa of angiosperms (Cuttler, [13,14; Stace, [90,91]; Vaikos, [97,98]. Very rarely trichomes are also found striated in case of *Celastrus paniculatus* and *Ventilago denticulata* (Shisode and Patil [75].

Hartog and Bass [29] studied leaf epidermal diversity of the Celastraceae (*sensu lato*). They investigated characters of stomatal types, occurrence of crystaliferous

epidermal cells and indumentum. These authors are inclined towards the inclusion of Hipporcrateaceae (*sensu stricto*) in the Celastraceae proper. They employed anatomical characters to arrive at a natural classification below and above generic level. This is dilated later. The present author's study (Shisode and Patil [75,77,83] of foliar trichomes shows that unicellular conical or cylindrical trichomes and simple filiform types are common to all the three families investigated. Few other types especially unicellular two-armed, unicellular bulbous, bicellular trichomes occur sporadically in combination with other types. No trichome type is characteristic for a family. The stellate scales are recorded only in case of *Pomaderris apetala* of the Rhamnaceae. This obviously helps earmark the taxa within the Rhamnaceae. Behnke [5] noted P-type and S-type of plastids present in angiosperms. According to him the family Rhamnaceae have S- type, whereas Vitaceae as well as Leeaceae have P-type. This evidence does not help visualise affinity between Rhamnaceae and Vitaceae *(sensu lato).* At the same time, it does not favour splitting of the latter into two separate families [84].

6. ULTRASTRUCTURAL EVIDENCE

Mennega [52] investigated wood anatomy of some Hippocrateaceae. In her opinion, the absence or presence of intraxylary phloem has no bearing with the systematic position of the species. However, she recognized two groups based on the wood structure. The first group, embracing *Anthodon Cuervea, Elachyptera, Hemiangium, Hippocratea, Hyleanea, Pristimera* and *Prionostemma,* can be characterised by broad and very high rays, mainly composed of procumbent cells, by the presence of septate fibres, tracheids and the absences of intraxylary phloem. The second group, consisting of *Cheiloclinium, Perristasa, Tontelea* and *Salacia,* is characterised by almost exclusively uniseriate rays, composed of square and upright cells, septate fibres in 2 to 5 cells wide concentric bands, intraxylary phloem of the foraminate or circumvallate type. She sheds more light on the genera thought 'intermediate' or 'links' between the Hipporcrateaceae and Celastraceae. *Cheiloclinium* is one such genus. Loesner [44] includes it in the Celastraceae. However, the wood anatomy of this genus evidently is in good agreement with species of *Salacia.* Another genus viz., *Campylostemon* kept under the Celastraceae by Loesner [44] was returned by Lawalree [42] to the Hipporcrateaceae because its wood exactly matches that of *Hippocratea* of the Hippocrateaceae. *Kokoona* sometimes thought *Celastraceous* belonged to the Hippocrateaceae. This is, however, contradicted by its very regular concentric parenchyma bands and by the absence of septate fibres. Stenzil [92] finds certain resemblance in petiolar anatomy of *Kokoona* to the genus *Maytenus.*

Mennega [53] studied some more genera of the Hippocrateaceae. *Campylostemon* is thought by some taxonomists as belonging to the Celastraceae or as intermediate between the Hipporcrateaceae and Celastraceae. In her opinion, the genus *Campylostemon* resembles closely in its wood anatomy to the Hippocrateaceae. Mennega *(op.cit.)* still adheres to the concept of Hippocrateaceae as a family of its own since no intermediate linking Hipporcrateaceae with Celastraceae. Moreover, She agrees with the

independent familial status - Celastraceae and Hipporcrateaceae assigned by Cronquist [11] and others. The division of the latter into two distinct groups, depending up on the nature of fruit also confirmed by her present wood anatomical studies. Halle [26,27] also accepts these two groups the Salacieae on one hand and Hippocrateae, Halictonemeae *and* Campylostemoneae on the other. According to him, genera of the *Salacieae* with drupaceous fruits and wingless seeds are anatomically characterised by a rather thin bark of uniform thickness, narrow rays, presence of septate fibres often arranged in a parenchyma like banded pattern, and by the frequent presence of intraxylary phloem. Other genera with dry dehiscent apocarpic fruits, an often winged seeds, have stems showing a thick bark deeply intruding to the woody cylinder, irregular or as regular deltoid intrusions, wide rays and intraxylary phloem lacking.

Zhang and Mennega [101] extended their wood anatomical observations on the basis of *Bhesa.* A comparison with other *Celastraceous* genera revealed that the combination of the salient wood anatomical features of *Bhesa* e.g. exclusively scalariform perforations, vessels mainly in radial multiples, large vessel-ray pits, non septate thick walled labriform fibres, fine apotracheal parenchyma bands, many-celled parenchyma strands and chambered prismatic crystals are unique within the family. Thus the genus *Bhesa* is an isolated one in the *Celastraceae*. An isolated position for *Bhesa* (previously named *Kurrimia)* was also advocated *(cf.* Hou, [35]; Metcalfe and Chalk, [54]) and to a position in the *Saxifragaceae (sensu lata) or* as a separate family *Kurrimiaceae* closer to the *Celastraceae*. A computer search of the GUESS wood identification database with information of over 500 wood species (Wheelar et.al.,[99] revealed that outside the *Celastraceae* the genus *Bhesa* matches rather closely with the tribe *Couleae* of the family *Olacaceae.*

Hallier [28] and Bentham Hooker-[7] included the genus *Hippocratea* in the *Celastraceae*. However, Engler and Prantl [16] Bessey [9] and Hutchinson [31] divided the *Celastraceae* into two independent families viz., the *Celastraceae* and Hippocrateaceae. There are evidence for and against the inclusion of *Hipporcrateaceae* with the *Celastraceae*. Smith [85] showed many differences between these two but emphasized on their closer affinities. Smith and Bailey [86] thought the division between the two families *Celastraceae* and *Hippocrateaceae* as artificial. Metcalfe and Chalk [54] have pointed out a very close relation between the two families on anatomical grounds. Erdtman [18] also noted great resemblance between the pollen grains of the two families.

7. WOOD ANATONICAL EVIDENCE

Ghosh and Shahi [22] *studied* wood anatomical properties especially of the two Indian genera *Rhamnus* and *Ziziphus.* According to them, these can be distinguished easily by the flame-like arrangement of vessels which is striking feature for species of *Rhamnus,* however, this totally lacks in the species of *Ziziphus.*

8. FLORAL ANATOMY

Floral anatomy of some genera of the Celastraceae has been attempted by Berkeley [8]. The families Rhamnaceae and Vitaceae have been also studied similarly by Nair and Sharma [63], Prichard [68], Kashyap [39,40]. According to Prichard (1955) the Rhamnaceae and Celastraceae have arisen from a hypothetical ancestor of obdiplostemonous stamens. The loss of antisepalous stamens resulted in the former, while the disapearance of the antipetalous whorl is noted in the latter. He also stated that there is very little in common floral anatomically in these two families. The floral anatomical features of the Rhamnaceae and Vitaceae such as antipetalous stamens, conjoint petal—stamen trace, conspicuous interstaminal disc and basically parietal placentation derived from axile condition hold them together (Nair and sarma, [63]; Prichard, [68]; Kashyap, [39].

9. EMBRYOLOGICAL EVIDENCE

Adatia and Gavade [1] studied embryology of some Celastraceae. They showed main similarities as well as differences in the embryology of *Hippocratea and* Celastraceae. The genus *Hippocratea* is similar to Celastraceae in glandular tapetum, anatropous and bitegmic ovoules, *Polygonum* type of embrosac, presence of endothecium and nucelar type of embryo sac, presence of endothecium and nuclear type of endosperm development. According to them, the genus *Hippocratea* differs from the Celastraceae in tenuinucellate ovules and exalbuminous, winged and exarillate seeds. They suggested disbanding of *Hippocratea* from the Celastraceae and the inclined to place in a separate family - the Hippocrateaceae but closer to the former. It appears from their account that there are more similarities of *Hippocratea* with the Celastraceae than their differences. This led to controversy in the taxonomic position of the genus. *Hippocratea* on the basis of their results. They considered *Hippocratea* belonging to the Celastraceae. Guadalupe [24] and Espinosa et al*.,* [19] extended embryological observations of some more species of *Hippocratea.* Their study showed similarity between *Hippocratea* and the family Celastraceae.

The Stackhousiaceae and Rhamnaceae have certain embryological similarities e.g. extension of funicular vascular strand beyond the chalazal, *Polygonum* type of embryo sac nucelar endosperm and Asterad type of embryo. The family Stackhousiaceae differs from Rhamnaceae in having a single anther middle wall layer, tenuinucellate ovules on basal placenta, absence of nucellar caps, single celled female archesporium, embryosac absorbing inner integument and seed coat formed by outer integument alone (Mauritzen, [50]; Narang, [64]. Embryologically, Rhamnaceae and Vitaceae share more common features e.g. anther wall consisting of five layers of cells, multinucleate Secretory tapetum, bitegmic crassinucellate antropous ovules with downwardly directed micropyle, thick integument, a nucellar caps, a well developed hypostase, free nuclear endosperm etc. (Dolchar, [15]; Kajale, [37]; Srinivasachar, [89]; Mulay, Nair and Sastry, [55]; Nair and Parasuraman, [60]; Nair and Nambisan, [59]; Nair and Suri, [62]; Kashyap, [38,40].

The Celastraceae differ from the Rhamnaceae in the presence of tenuinucellate or weakly crassinucellate ovules, absence of integumentary vascular tissue and hypostase, fusion of polar nuclei before fertilization. Solanad type one embryo; seeds arillate and their spathulate type of internal morphology (Adatia and Gavade, [1]; Martin, [49]. Hutchinson [31] included the family *Elaeagnaceae* along with Rhamnaceae and Vitaceae in his order Rhamnales. Therefore the comparison of Elaeagnaceae and Rhamnaceae appears pertinent. Both of them share similar embryological features e.g. anatropous, bitegmic and crassinucellate ovules, nuclear endosperm and investing type of internal morphology of the seed (Martin, [49]; Sarma, [70]. Rhamnaceae differs from Elaegnaceae *i*n absence of integumentary vasculature and parietal tissue in the ovule, polar nuclear fusion before fertilization, one antipodal cell becoming prominent and persistent, presence of chalazal embryosac, haustorium and spathulate type of internal morphology of the seed (Martin *loc. cit;* Sarma *loc. cit.)* The family Rhamnaceae and Vitaceae are generally placed in one taxonomic entity. They share similar embryological features such as anatropous, bitegmic, crassinucllate ovule, formation of nucellar cap, presence of hypostase *Polygonum* type of embryo sac, nuclear endosperm and *Asterad* type of embryo (Adatia *et.al* [1,2], Kashyap, [38,40], Mulay *et.al.* [55]; Nair and Bajaj, [57]; Nair and Parasuraman;[60] Nair and Suri, [62];Souges [88] These families exhibit striking embryological dissimilarties . Integumentary vascular strand, epistase and *Allium* type of embryo sac noticed in Rhamnaceae, obturator, proliferation on nucellar tissue and its disappearance during post fertilization stages, haustrial nature of embryo sac and ruminate endosperm observed in *Vitaceae* are not encountered in the Rhamnaceae (Adatia, *loc.cit.;* Kashyap, *loc.cit.,* Mulay, loc.cit., Nair and Bajaj, *loc.cit.,* Nair and Parsuraman, *loc.cit*., Nair and Suri, *loc cit*, Souges *loc. cit.*) [71,72].

A resume of taxonomic alignments of different authors points out that the family Vitaceae and Celastraceae (including Hippocrateaceae) are kept in the same taxonomic group. Therefore it appears, pertinent to discuss their taxonomic affinities on embryological ground. They are similar in having *Polygonum* type of embryo sac, nuclear endosperm and similar structure of anther wall. The weakly crassinucellate ovules with one layer of parietal tissue, outer integument forming the micropyle, the inner integument getting, absorbed by the embryo sac, absence of hypostase and *Solanad* type of embryo are characteristic of the Celastraceae (*sensu lato*). This set of embryological characters are absent from the Vitaceae. It is to be further noted that ruminate endosperm, perichalazal growth, ingrowth from seed coat and few other developmental features of seeds which are characteristic of Vitaceae are absent in the Celastraceae (*sensu lato*).

10. PALYNOLOGICAL FEATURES

The commonly occurring 3-colporate or 3-colporoidate condition of pollen grain is found in the families such as Celastraceae, Hippocrateaceae, Stackhousiaceae, Salvadoraceae, Staphyleaceae, Aquiofoliaceae, Empetraceae, Cyrillaceae and Goupiaceae. The family Siphonodontaceae is characterized by 3- porate pollen

grains. The pollen grains in Corynocarpaceae are not encountered in the Celastraceae and other families enlisted above. The family Icacinaceae is, however, eurypalynous. These three families therefore, appear not related on pollen morphological ground to the families of celastralean plexus (*cf*. Erdtman [19], Farzana and Bhandari, [20].

11. SYNTHETIC ASSESSMENT OF HIGHER HIERARCHY

The Family Staphyleacae has been placed under the order Sapindales by Engler and Diels [17]; Hutchhinson [31,33,34]; Benson [6]; Cronquist [10,11,12]; Takhtajan [95]; Soo [87], whereas it is referred to order Celastrales by Melchior [51]; Rendle [69]; Bessey [9] and Wettstein [100]. The Staphyleaceae are included in the Sapindales by Bentham and Hooker [7]. The anatomical characteristic such as anisocytic stomata, vessel end with scalariform perforation plate, clustered crystals and paratracheal wood parenchyma do not help disband the family Staphyleaceae from the other core families of the *Celastralean* plexus. Hallier [28] refers it to the Rosales and conceived it allied to the Cunoniaceae and Saxifragaceae.

According to Inguva [36] the family Sapindaceae including Staphyleaceae is homogeneous in possessing similar flavonoid profile. She also confirms origin of these two from a common stock. As pointed out earlier the 3-colporate condition of the pollen grains in Staphyleaceae does not preclude the affinity with the *Celastralean* families, although similar condition is also noted in the families of the order Sapindales. Erdtman [18] allies it with the Celastraceae. Foster [21] on cytological ground inferred that they have a common origin with Aceraceae. Cronquist [11] considers the Staphyleaceae as anomalous under the Celastrales because of compound leaves; he thinks the Staphyleaceae as intermediate between the Cunnoniaceae (Rosales) and the Sapindaceae and Aceraceae (Sapindales). He also refers Staphyleaceae to the order Sapindales. The Staphyleaceae is indistinctive on account of cup like intrastaminal disc. numerous ovules, ample endosperm and straight embryo. It is also distinguishable by absence of ellargic acids not cyanogenic not saponiferous and in lacking iridoid compounds. In macromorphological features like pinnate stipulate leaves and ovules numerous in each locule render the Staphyleaceae anomalous in the order Celastrales. However, it is to be noted that the micromorphological characteristic e.g. anisocytic stomata, vessels with scalariform perforation plates, clustered crystals, paratracheal wood parenchyma, 3-colporate pollen grains, ovule anatropous, bitegmic, crassinuecellate, endosperm nuclear tapetum glandular, simultaneous cytokinesis in M.M.C. Pollen grains 2-celled at anthesis, fusion of polar nuclei prior to fertilization and Polygonum type of embryo sac etc. Decisively allies the Staphyleaceae with the Celastrales rather than the Sapindales. However, it should be regarded the most primitive family of the order Celastrales.

A resume of different systems of Ranalian and Englerian schools indicate that the families *viz.*, Celastraceae. Hippocrateaceae, Stackhousiaceae, Salvadoraceae, Staphyleaceae, Siphonodontaceae, Icacinaceae, Goupiaceae,

Empetraceae, Cardiopteridaceae and Cyrillaceae constitute core of the Celastrean plexus (Shisode and Patil [82].

The families *viz*., Celastraceae (*sensu stricto*) Aquifoliaceae Cyrillaceae, Icacinaceae, Staphyleaceae, Empetraceae, and Goupiaceae, show similar development in vessel specialiazation. The end walls of vessel in these families exhibit scalariform perforation. However, in the families Hippocrateaceae, Salvadoraceae, Stackhousiaceae and Corynocarpaceae, the end walls of vessels are simple perforated and advanced over the other celastralean families.

The family Celastraceae-Hippocrateaceae complex exhibit a variety of stomatal types ranging from anomocytic to anisocytic to paracytic to heliocytic, cyclocytic, etc. Few genera like Kurrimia (Bhesa) Brassiantha Xylonimus, Hedrainthera however show exclusively paracytic condition. In remainder of this alliance other types noted earlier are found in different combinations, although the anomocytic type is more prevalent in this alliance. The core families of the Celastrean plexus *viz*., Staphyleaceae, Goupiaceae, Cyrillaceae, Auquifoliaceae, Icacinaceae, Stackhousiaceae also show anomocytic or anisocytics as dominant or codominant types. It is only Corynocarpaceae and Salvadoraceae which have paracytic stomata. The Siphonodontaceae are also marked out by the laterocytie type. The laterocytic condition is sometimes thought as “complex anisocytic” (Hartog and Bass, [29].This resume of the stomatal features in Celastralean plexus indicates that all these families can be easily accommodated under one taxonomic entity. The various other types noted particularly in the Celastraceae (sensu lato) are derivable from one another (*cf.* also Hartog and Bass, *loc. cit.*). Majority of celastralean families show unicellular and uniseriate trichomes. The other types such as stellate, peltate scales or hairs are rarely noted in few taxa. The typology of trichomes certainly helps to lump all these families under one taxonomic entity.

According to Gibbs [23] raphides are absent in the order Celastrales including the family Rhamnaceae. Their prominence, in this opinion, is notable in the allied family Vitaceae *(sensu lato)* However, it is interesting to note that majority of these families have crystalliferous foliar epidermal cells. The crystals are either solitary on clustered. This also evidences to treat the various families of this plexus under one broad taxonomic rank.

The wood anatomical features, especially the distribution of wood parenchyma do not exclude close affinities of these families from one another as both type viz., paratracheal and apotracheal are observed in different families of the alliance as also within the same families. The common occurrence of anomocytic stomata, unicellular or uniseriate hairs, solitary and clustered crystals, wood parenchyma paratracheal, vessels being either simple or scalariform perforated do not preclude the affinities of the Rhamnaceae and Vitaceae *(sensu lato)* Instead these features help align all these families closely.

Bentham and Hooker [7] placed the family Stackhousiaceae under the order Celastrales in between the families Celastraceae and Rhamnaceae. Engler and Prantl [16] however kept it under the order Sapindales in between the families Salvadoraceae and Staphyleaceae. Hutchinison [30] followed the same

treatment. The family Hippocrateaceae is said to form a transitional group between the Stackhousiaceae and Celastraceae (*sensu stricto*). Mautrizen [50] Narang [64] investigated embryology of the genus Stackhousia (Stackhousiaceae) and pointed out many similarities with the Celastraceae. In either of the families, the outer integuments form the micropyle, while the inner integuments are consumed by the embryo sac; the nucellus degenerate at the 2-nucleate and 4-nucleate stage of embryo sac; the embryosac is monosporic and eight nucleate; the endosperm is nuclear. The author therefore lends support to assign the family Stackhousiaceae to the order Celastrales. The further remarked that the family Stackhousiaceae is the closest to the Celastraceae and the Hippocrateaceae.

The relationship of the family Salvadoraceae and the order Celastrales has been the subject of dispute in the past. Bentham and Hooker [7] and Bessey [9] assigned it to the Gentianales. Rendle [69] placed it under the order Oleales. Engler and Diels [17] placed the Salvadoraceae in polypetalae in Celastrineae under the Sapindales. Hutchinson [34] recommended similar taxonomic position. Gunderson [25] considered it within the order Celastrales. Takhtajan [95] and Cronquist [11] followed the same treatment. Takhtajan (loc. cit) accepted its close relationship with the Celastraceae.

Maheshwaridevi [45,47] studied embryology of Salvadoraceae as well as the families of Gentianales. Johri reviewed and assessed the embryological features of Salvadoraceae the Gentianales and Olacales. He opined that the inclusion of Salvadoraceae in either of the order is not justifiable. On the contrary, in his opinion, the inclusion of Salvadoraceae in the order Celastrales is best on the basis of embryology. The representative genera viz. Azima and Salvadora of the Salvadoraceae share the features with the Celastraceae such as two - celled pollen grains, anatropous bitegmic crasinucellate ovules, Formation of parietal tissue and nuclear type of endosperm. The placement of Salvadoraceae in the Celastrales is also supported by floral anatomy (Kshetrapal, [41]. Gamopetaly in case of Salvadora seems to contradict this position but should be regarded as an exceptional feature for the family. This view is also impressed by Melchior [51].

Erdtman [18] in the light of palynological evidence available opined that pollen grains of the Celastraceae are more or less similar to those in Hippocrateaceae. Pollen grains are usually three-colporate in both families. He drew attention towards the artificial division between these families as pointed out by Smith and Bailey [86]. Likewise, Erdtman (*loc. cit.*) pointed out pollen morphological similarly between the families Rhamnaceae and Vitaceae. He regards Rhamnaceae as a stenopalynous family. Pollen grains are usually three colporate. It is also to be noted that the pollen grains in the Celatraceae and Rhamnaceae are three colporate but the former have usually more rounded pollen grains. The grains are triangular in the Rhamnaceae.

Farzana and Bahandari [20] investigated the pollen morphology of the Indian family Rhamnaceae They also concluded that the family Rhamnaceae is a stenopalynous one. They observed pollen grains usually three zonicolporate, to sub - oblate sphaerodial and often triangular. Exine strarification is more or less

reticulate and psilate. They opined that there are only minor pollen morphological differences which are not of much taxonomic significance. According to them the occurrence of similar pollen grains in the Rhamnaceae and Vitaceae is suggestive of their close affinities as reported by Erdtman (1952) They further suggest close affinities between the three families viz. Rhamnaceae, Vitaceae and Celastraceae on palynological base.

Lobreau (1971) divided the order Celastrales on the basis of pollen morphology into two groups – the first group consisting on the families viz.,; Celastraceae, Hippocrateaceae, Staphyleaceae and Stackhousiaceae characterized by pollen grains with a recticulate sculpturing and simple endexine, whereas the another group comprising the families such as Iccacinaceae, Siphonodonataceae, Cordiopteridaceae, Salvadoraceae and Aquifoliaceae characterized by the pollens with various sculpturing patterns and an irregularly cracked, and structural endexine. He considers some families of the order as doubtful in position and remarks for their exclusion from the Celastrales.

Muller [56] recorded fossil angiosperm pollen types of different families. Based on this evidence, he gave the appearance of angiopermous families. The families *viz.*, Celastraceae (*sensu lato*) Rhamnaceae and even Vitaceae appeared in the oligocene of teritiary. This indicates that none of them originated from the other. On the contrary, this evidence warrants their origin from a common ancestrous stock. The Sapindales into which Celastraceae is sometimes included (Engler and Diels, [17] appeared quite later in the upper miocene of the tertiary. This evidentlty demonstrates that none of the Sapindales are progenitors of the Celastrales.

A resume of the basic chromosome number (x) of the three families presently under consideration brings certain things to the fore. The base numbers in the Celastraceae and Hippocrateaceae are not by far suggestive of their relationship to each other. More studies on other taxa of these families are obviously needed for better comprehensive treatment and phylogenetic considerations. However, the close relationship between the Rhamnaceae and Vitaceae appears certainly warranted. The base numbers x = 10, 11, 12, 13 are commonly met with the genera, of both families. Other base numbers in rest of the members of these families also appear derivable from them. Thus their closer affinity may be plausibly pointed out.

Gibbs [23] while summarizing chemistry of Celastraceae and Hippocrateaceae commented that very little of the chemistry is known of the latter and hence expressed inability to assess the affinity between them. He noted (doubtfully) the absence of raphides in the Celastraceae. However, the present author noted them in Euonymus fortunei. He also doubted occurrence of raphides in the Hippocrateaceae. The present author also could not locate them in the species studied. It is interesting to note that three specues of Kurrimia (now the genus *Bhesa*) are said to be accumulators of aluminium. No accumulates of aluminium are known within the other celastraceous members and in the Hippocrateaceae. Rhamnaceae and Vitaceae as well. Gibbs [23] noted absence of raphides in the

Rhamnaceae, present study also records their absence in the family. However, they have been noted in the members of Vitaceae. The chemical evidence then available to Gibbs [23] hardly reflects relationship of the Rhamnaceae to the Celastrales.

Plouvier [67] noted distribution of aliphatic polyals and cyclitols in the Celastraceae and Hippocrateaceae. On the chemical basis, he confirms the parentage of Hippocrateaceae with the Celastraceae. He further adds that the family Scrophulariaceae has affinities with the Celastraceae and Hippocrateaceae through the intermediate family Stackhousiaceae.

Inguva [36] studied phytochemistry and systematics of some Sapindales and its related taxa. According to her, flavonols and proanthocyanidins form the major phenolic pigments of the group. Flavonols are particularly abundant in Celastraceae as well as in Rhamnaceae. Likewise alkaloids are also abundant in these two families. The Origin of the orders *viz.*, Celastrales (Celastraceae), Rhamnales (Rhamnaceae) and Vitidales (Vitaceae and Leeaceae) is according to her unconfirmed. The Staphyleaceae, which are sometimes included in the order Sapindales, show closer affinities to the Celastrales, flavonols, quinones and parenthocyanidins are hightly prevalent in the Rhamnaceae. Flavonols and glycoflavonos are rare but these characters according to her make the family a natural taxon. The family Rhamnaceae is however distinct in producing quinines and peptide alkaoids.

The Vitaceae and Leeaceae are dissimilar; the former shows the presence of flavons, glycoflavones and flavenois, whereas the latter produces highly hydroxylated compounds like myricatins and gallic acid. Both these families do not synthesize the quinones and peptide alkaloids characterstic to the Rhamnaceae. She therefore opposed grouping of the Vitaceae and Leeaceae alongwith the Rhamnaceae under the order Rhamnales.

The genus *Leea* is treated as a separate family–the Leeaceae between the Vitaceae (Sarmentaceae) and Meliaceae, Sussenguth, [93], Barting, [4]. It is also supported by Melchior [51]; Cronquist [10,11,12] Takhtajan [94,95] and Wettesein [100] treated it as a subfamily Leeoideae of the Vitaceae. The genus Leea shows many embryological similarities with the Vitaceae. Both of them exhibit multicellular microsporial archesporium, bitegmic crassinucleate ovules with a cap, presence of hypostase, Polygonum embrayo sac, perichalaza, nuclear endosperm, Asterad type of embrayo development, structure of seed coat, fatty reserves in endosperm etc. (Adatia et al. [1,2]; Mulay et al. [55]; Kashyap, [38,39]; Nair and Bajaj, [57]. Nair and Parasuraman, [60,61]; Nair and Suri [62]; Periaswamy, [66]. They also show similar floral anatomical features such as valvate sepal and petals, antipetalous, stamens, fertile introrse anthers, conjoint petal- stamen traces, gynoecium derived from multicarpellary condition (Kashyap, [39]; Nair and Mani, [61]; Nair and Nambisan, [59] Both of them have tricolplate pollen grains (Erdtman, [18]. They are similar in anomocytic stomata, presence of raphides and mucilage, vessels with simple perforations, and paratrachel wood parenchyma, similar trichomes and deciduous pearl glands

(Metcalfe and Chalk,[54]. In the broad medullary rays and petiolar anatomy, *Leea* is different from the other Vitaceae (Metcalfe and chalk [54]. As stated earlier, chemically they are dissimilar (Inguava 1990) Leea is also different from the remainder Vitaceae in the erect habit. Terminal inflorescence, exstipulate, leaves, petiolar anatomy of divided strands, epipetalous stamens, staminal tube, inner whorl of staminodes, obdiplostemonary condition and absence of disc. 4-6 lobed ovary, development of false septa in the ovary, single ovule per loculus, chalazal, ingrowth in the seed, absence of raphides in seed coat (Kashyap [39]; Nair, [61]; Nair and Mani, [60].

12. CONCLUSION

Authors consulted a vast array of Cleastrlean taxa. They also brought Informetion from different disciplines plant science to the fore front. Earlier research workers evaluated this alliance in isolation using a single discipline. It is, therefore, the taxonomic boundaries cris0 crossed time again. However, the present authors assessed all the disciplines comparatively to arrive at concrete conclusion regarding phylogeny and taxonomy of this Celastralean plexus. The authors thought that multidisciplinary approach is a drive necessity while assessing such complex alliance.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 6
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Pathological and Immunohistochemical Studies on Horn Cancer in Bovines

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ABSTRACT

Background: An investigation was carried out on twelve clinical cases of neoplasm of horn in bovines of Durg, Dhamtari and Rajnandgaon districts of Chhattisgarh suspected of bovine horn core carcinoma (squamous cell carcinoma) revealed the cytology, pathomorphology and immunohistochemical (IHC) expression of Pan-cytokeratin (Pan-CK), p53 gene, epidermal growth factor receptor (EGFR) and p16 gene in tumourous growth at horn in bovines.
Results: Eight out of 12 cases (66.66%) were confirmed as SCC of horn on the basis of histopathological and immunohistochemical analysis. Cytological examination of tumours by Papanicolaou staining revealed variation in shape and size of cells and altered nuclear details. Grossly neoplasms of horn revealed unilateral large cauliflower like growths at the base. SCCs were classified as well, moderately and poorly differentiated types on the basis of histopathology and immunohistochemistry (IHC). Well differentiated SCCs (n=4; 50%) were characterized by severe keratinization of horn epithelium with concentric arrangement forming keratin pearls also called as “cell nests”. Tumour islands of irregular shape observed in the horn epithelium invaded deep into dermis layer. Moderately differentiated SCCs (n=2; 25%) characterized by small keratin pearl formations and mitotic figures. Poorly differentiated SCCs of horn (n=2; 25%) revealed absence of distinctive keratin pearls although deep invasion from primary site was observed. SCC of horn revealed strong immunohistochemical

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staining of Pan-CK, p53 and EGFR and negative to p16. Highest immunohistochemical expression was observed in Pan-CK which confirmed the tumours were of epithelial origin and EGFR immunoexpression was confirmatory for malignancy and degree of metastasis. Neoplasms were confirmed as SCC by immunoexpression of Pan-CK, EGFR and p53 in malignant tumours including both well and poorly differentiated SCC of horn.

Keywords: Cell nests; EGFR; immunohistochemistry; keratin; papaniculaou; pan-CK; p53; p16; squamous cell carcinoma.

1. INTRODUCTION

Bovine horn core carcinoma also called as Squamous Cell Carcinoma (SCC) of horn is one of the most common cancer capable of metastatic spread and is observed in various forms across many animals [1,2,3]. It is malignant neoplasm of epithelial origin capable of metastatic spread so reffered as carcinoma. The accumulation of genetic and epigenetic alterations (stable and heritable alterations in gene expression and cellular function without changes to original DNA sequence) in cancer cells provides them with unwanted proliferative and metastatic potential. Squamous Cell Carcinoma of horn, also known as horn cancer, is a prevailing type of cancer in cattle especially *Bos indicus*. Horn cancer is generally unilateral and is encountered in cattle between 5-10 years of age [4]. In India, horn cancer affects approximately 1% of the cattle population and accounts for 83.34% of total tumours reported [5]. Horn cancer is a sporadic, malignant neoplasm affecting the horn core epithelium and predominantly seen in aged zebu bullocks and rarely in buffaloes [6,7]. The disease is associated with chronic irritation of the horns at their base by yoke [8] It usually fits behind the horns causing irritation to the horns and due to striking of the horns (mechanical injury) by yoke while ploughing eventually leading to horn core carcinoma. The bullocks appear to be highly susceptible as compared to bulls and cows. It is one of the most commonly encountered neoplastic conditions of economic importance in zebu bullock [9].

Cytokeratin is one of the most important tumour markers for diagnosis of squamous cell carcinoma, high variations in expression patterns of cytokeratins have been correlated to different pathways of epithelial differentiation leading to the accurate diagnosis and classification of tumours of epithelial origin into different subtypes by immunohistochemistry [10]. The expression of simple epithelial or non-cornifying stratified squamous epithelial cytokeratins in cutaneous SCCs may be a marker for their capability of invasion and metastatic potential [11]. Mutations in the p53 gene may cause cancer cells to grow and spread in the body. The inactivation of this gene possibly results in oncogenesis [12]. SCCs commonly have mutations in p53, and positive immunolabeling for p53 has been reported in animals especially in SCCs of non-pigmented skin secondary to exposure to UV radiation [13,14]. The tumour suppressor gene p16 has gained widespread importance in cancer, frequent mutations and deletions of p16 in human cancer cell lines first suggested an important role for p16 in carcinogenesis [15]. SCCs have been shown to express p16 through

immunolabeling. Antibodies targeting p53 and p16 have been used as prognostic factors in SCCs. Epidermal Growth Factor Receptor (EGFR) is a key factor in epithelial malignancies, and its activity enhances tumour growth, invasion and metastasis [16]. EGFR plays an important role in maintaining normal cell function, dysregulation of EGFR signaling towards malignancy due to effects on cell cycle progression, inhibition of apoptosis, induction of angiogenesis and promotion of tumour cell motility and metastasis [16]. Occurrence of horn core carcinoma is sporadic in field conditions in Chhattisgarh, so keeping that in view, the study was undertaken with the following objectives:

To study the cytology and histopathology of squamous cell carcinoma of horn in affected bovines

To detect horn core carcinoma through tumour biomarkers (Pan-CK, p53 gene, EGFR and p16 gene) using immunohistochemical technique

2. MATERIALS AND METHODS

The study was conducted in the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Anjora, Durg (Chhattisgarh) to explicate the cytology, pathology, and immunohistochemical alterations in Squamous Cell Carcinoma of horn in bovines. The samples were collected from various gaushalas, dairy farms, teaching veterinary clinical complex (TVCC, Durg), College of Veterinary Science, Anjora and government veterinary hospitals (GVH) of Durg, Dhamtari and Rajnandgaon districts of Chhattisgarh (Table 1). Tumour biomarkers such as p53 (Tumour suppressor gene), p16 (Tumour suppressor gene), Pancytokeratin (Pan-CK) and Epidermal Growth Factor Receptor (EGFR) in tissues were investigated by immunohistochemical technique. The study was conducted over a period of 6 months from January to June 2022. All the experimental procedures were carried out as per recommendations of the Institutional Animal Ethics Committee (IAEC).

Cytological studies: Cytology samples/ tissue impression smears were processed and stained as per Papanicolaou staining procedure.

Papanicolaou staining: Staining techniques used to stain the cytological smears were as per the method by Papaniculaou and Traut [17].

Rapid-Pap nuclear staining: Papanicolaou staining was done using RAPID-PAP Kit. The staining procedure was followed as per manufacturer‘s instructions.

Papaniculaou staining (Pap EA-36 and Pap OG-6): Papanicolaou’s staining was used for cytological / impression smears for cancer cells and staining for keratin as per the method prescribed by Doddagowda et al. [18] and Raju, K. [19] and following stains were used:

Table 1. Details of collected samples suspected of bovine horn core carcinoma from different districts of Chhattisgarh

S No.	Case ID	Age	Sex	Breed	Location of neoplasm	District
1.	**BovHC1**	7 years	F	Kosli	Base of right horn (unilateral)	Dhamtari
2.	**BovHC2**	6 years	F	Haryana	Base of right horn (unilateral), extending deep to frontal sinus.	Dhamtari
3.	**BovHC3**	4 years	F	Non-descript	Base of left horn, extending to frontal sinus and skull	Durg
4.	**BovHC4**	10 years	M	Non- descript	Base of left horn	Dhamtari
5.	**BovHC5**	9 years	M	Non- descript	Base of right horn	Durg
6.	**BovHC6**	8 years	M	Non-descript	Base of right horn	Durg
7.	**BovHC7**	7 years	M	Haryana	Base of right horn	Dhamtari
8.	**BovHC8**	5 years	F	Non-descript	Base of left horn	Rajnandgaon
9.	**BovHC9**	11 years	M	Kosli	Base of right horn	Rajnandgaon
10.	**BovHC10**	8 years	F	Non-descript	Base of right horn	Durg
11.	**BovHC11**	10 years	F	Non-descript	Base of left horn	Durg
12.	**BovHC12**	2 years	M	Non-descript	Base of right horn	Durg

Table 2. Tumour markers and primary antibodies used in immunohistochemical study

Tumour markers	Antibody	Clone	Catalogue no.	Make	Lab animal in which Ab raised with Ig class	Volume
Pan-Cytokeratin (Pan-CK)	Cytokeratin Pan Plus	AE1 and AE3	MSG098	Zytomed system GmBH, Anhaltiner-stranbe 16, 14163 Berlin, Germany.	N/A	6ml Conc., RTU
p53	Anti- p53	D07	AM239-10M	BioGenex Lab., Fremont, California (CA 94538) USA.	Mouse, Ig class: IgG1	10ml, RTU
Epidermal Growth Factor Receptor (EGFR)	Anti-EGFR	Polycl-onal	AM335-10RE	BioGenex Lab., Fremont, California (CA 94538) USA	Rabbit, N/A	10ml, RTU
p16	Anti- p16	G175-405	AM540-10M	BioGenex Lab., Fremont, California (CA 94538) USA	Mouse, Ig class: IgG	10 ml,RTU

RTU: Ready to Use, N/A: Not Available

Procedure: Impression smear were fixed with methanol biofix spray, slides were successively submerged in descending grade of alcohol 80%, 70%, 50% and water for one minute each. Slides were then stained with Harris Hematoxylin solution for approximately 5 min. Haematoxylin stained slides were immersed in water 6 times for 1 sec., submerged in 0.5% Hydrochloric acid, 8 times for 1 sec. Slides were rinsed with tap water for 5 minutes, and passed through ascending grades of alcohols, 50%, 70%, 80% and 96% for 30 seconds in each of them. Slides were then stained with Pap OG(Orange Green)-6 for 1-1.5 min. Excessive dye was washed in two 96% ethanol baths twice by immersing the preparation 2 times in each for 3 to 4 sec., Ethanol washed slides were stained with Papanicolaou EA(Eosin Azure)-36 for 1.5-2 min. Pap stained slides were washed in 3 different containers of ethanol 96% v/v by immersing the preparation 2 times of 3-4 seconds in each of them and washed in absolute ethanol for 30 sec. Alcohol washed slides were immersed in the preparation of bath of xylene, mixture of isomers and absolute ethanol for 4 min. Slides were then rinsed with xylene and mixture of isomers by immersing the preparation for 3 min. in a bath and finally mounted with DPX mountant.

Pathological studies:

Gross pathology: Gross morphological features of tumours like location, shape, colour and consistency of the tumour were examined. Tumours observed at horn were suspected to have been squamous cell carcinomas on the basis of gross pathology. Unique case identity (Case ID) was given to each sample [35].

Histopathological examination: The formalin fixed tissues were cut into small pieces of 2-3mm thickness and washed thoroughly in tap water overnight, dehydrated in graded alcohol or acetone series, cleared with graded xylene and embedded in paraffin wax. The paraffin embedded tissue was cut in 3-5µ thickness. The sections were deparaffinised in xylene and stained with Haematoxylin and Eosin (H&E) stain and mounted with DPX [20]. Stained sections were examined under light microscope. Tissue samples fixed in 10% formalin were processed for histopathological examination and stained as per standard H&E method of staining. The sections were examined microscopically for histological changes.

Immunohistochemical studies: Preliminary diagnosis of squamous cell carcinoma was made on the basis of clinical examination and gross findings. Later on Histopathological examination was carried out on 12 samples suspected of bovine horn core carcinoma, out of which 8 selected tissue samples evident of SCC were processed for immunohistochemistry (IHC) to detect biomarkers such as pancytokeratin (Pan-CK), p53, Epidermal Growth Factor Receptor (EGFR) and p16 and malignancy of tumours. Tumour biomarkers were examined and estimated in tissues by immuno-histochemical technique.

Source of Materials: Tissue samples were processed for immunohistochemistry (IHC) to detect biomarkers such as Pan-CK, p53, EGFR and p16 with reference

to Kumar et al. [12] at Dr Lal PathLabs, PathVets Veterinary Diagnosis, Chittranjan Park, New Delhi (110019).

Tissue processing for immunohistochemical staining: Tissues in 10% neutral buffered formalin solution and stored for 72 hours. Tissue dehydration with increasing concentration of ethanol with 50%, 80%, 90% and absolute alchohol respectively for 1 hour each followed by treatment with acetone for 30 minutes and clearing of tissue by xylene. Tissues were embedded in paraffin wax (Leica Microsystem, Paraplast tissue embedding medium) at 65°C. For further processing, 4-5µ sections were cut using a rotary microtome. Tissue sections were then put on the slides and stored until further use.

Immunohistochemical staining: Deparaffinization, antigen retrieval and immunolabeling of sections were carried out in automated immunostainers. Immunohistochemical labelling for all markers (p53, p16, Pan-CK and EGFR) were carried out on the Bench Mark Automated Staining System (Ventana Medical systems, Inc.). Antigen retrieval was performed for 60 minutes using Ventana Medical Systems Retrieval Solution CC1 according to the method prescribed by Fornazari et al. [21]. Antigen retrieval, blocking with bovine/goat serum albumin to avoid unwanted secondary binding of antigen and antibody. Immunolabeling of sections were carried out in automated immunostainers. Slides after incubation of chromogen substrate conjugated antibody at definate time-temperature combination were finally counterstained with haematoxylin. The stained slides were examined under light microscope.

Immunohistochemical Scoring (IHS): Immunohistochemical scoring was performed by estimating the percentage of positive cells and labeling intensity given in Table 3 as per the method described by Baghla et al. [22].

3. RESULTS AND DISCUSSION

Cytological Study: Cytological examination of tumours by rapid Papanicolaou nuclear staining revealed variation in shape and size of cells, as well as altered nuclear details (Figs. 1 & 2). Papaniculaou (EA-36 and OG-6) stained cells from malignant tumours revealed pleomorphism, characterized by significant variation in shape of the cells and anisocytosis in SCC of horn (Fig. 2), cluster of cells with great variation in cell sizes (Fig. 3). Other major findings observed were anisokaryosis, characterized by variation in the size of the nucleus, multinucleated cells were observed (Figs. 3 & 4) with minute deep-purple granules were also seen in the cytoplasm (Fig. 4).

Table 3. Basis for immunohistochemical scoring of tumour cells

Result	Score
Non immunoreactive	0
Immunoreactive in 1-25% cells	1+
Immunoreactive in 26-50% cells	2+
Immunoreactive in 51-75% cells	3+
Immunoreactive in 76-100% cells	4+

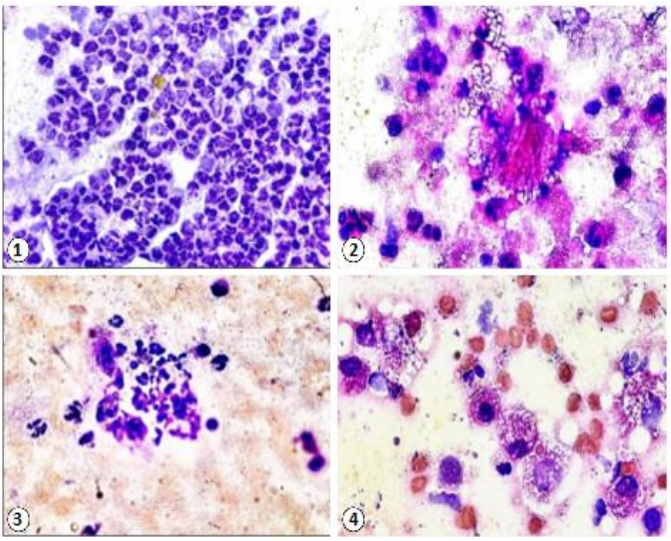

Fig. 1. Hyperchromatic and pleomorphic tumour cells (Rapid PAP X 400); Fig. 2. Significant pleomorphic cells and anisocytosis in SCC of horn (PAP EA-36 and OG-6 X 1000); Fig. 3. cluster of cells with great variation in cell sizes(Pap EA-36, OG-6 stain X 1000); Fig. 4. large sized tumour cells with giant nucleus (megakaryosis) in OSCC (BovEC3; Pap EA-36, OG-6 stain X 1000)

Cytological findings such as hyperchromasia, anisonucleosis, and multinucleated cell formation were in accordance with Hoffmann et al., (1978) and increased nuclear-cytoplasmic ratio and deep purple granules in the cytoplasm of cells in SCC were also reported by Garma–Avina [23].

Gross pathology: A total of 12 unilateral neoplastic growths at horn suspected of SCC were examined grossly, which revealed large, irregular masses at the base of horn. Cauliflower like growth at the base of horn were observed in most of the cases (n=9; Figs. 5 & 7). Two cases of large cauliflower like mass of about 20 cm diameter were seen (Figs. 5b & 7). Most of the tumours were soft in consistency and friable followed by firm consistency, solid nodular growth (Fig. 9 a), surface of most tumours were rough and verrucous (Fig. 8) with poor demarcation. Cut surfaces of tumours were whitish yellow, greyish white, greyish red to light brown in colour with widespread haemorrhages and areas of necrosis (Figs. 5 b & 7). Ulcerative masses with foul smelling purulent discharge were also observed in few cases (Fig. 9 b).

Fig. 5a. Large cauliflower like growth on the base of left horn in bullock (Case ID: BovHC4); b. Tumourous mass of about 20 cm diameter from the base of the horn in bullock (Case ID: BovHC4); Fig. 6a. Large cauliflower like growth on left horn of cow (Case ID: BovHC3); b. Soft and friable cauliflower like growth at the base of left horn having rough and verrucous surface (BovHC3); Fig. 7. Large cauliflower like growth about 20 cm diameter on the base of right horn in bullock (Case ID: BovHC10); Fig. 8. Solid nodular growth at the base of horn with firm consistency having rough and verrucous surface (Case ID: BovHC11); Fig. 9 a. Firm nodular growth on the base of right horn of Haryana cow (Case ID: BovHC2); Fig. 9 b. illustrating foul smelling purulent discharge on the base of right horn (Case ID: BovHC2)

Gross pathological findings of neoplasm of horn like unilateral growth observed in all cases examined was in accordance with Kalim et al. [24] Pink cauliflower like soft, friable growth with bleeding at the base of the horn were also reported by Giri et al. [25], Kumar et al. [26] and Sharma et al. [10], Rough and verrucous surface of the tumourous growth was also reported by Reddy et al. [27]. Foul smelling purulent discharge observed in the present study was also reported by Kumar et al. [26]. Firm consistency of tumours and scattered growth with poor demarcation observed in the present study was also reported by Baniadam et al. [28].

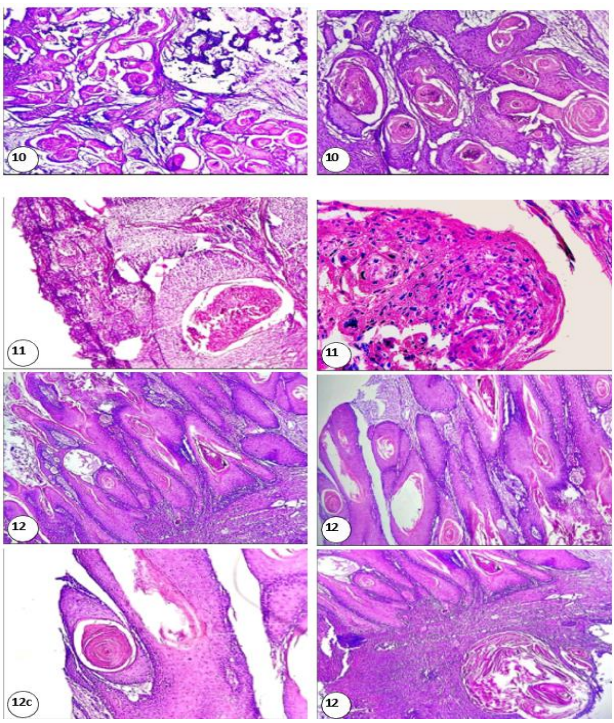

Fig. 10a. Well differentiated SCC of horn depicting formation of numerous epithelial pearls with mineralization (Case ID: BovHC6; H&E X 40); Fig. 11 b. Excessive keratinization of horn epithelium with concentric arrangement forming keratin pearls / Cell nests (Case ID:BovHC6; H&E X 100); Fig. 11a. Moderately differentiated (Grade II) SCC showing large keratin pearl differentiated SCC (BovHC1; H&E X 100; 11 b) H&E X 400); Fig.12a): Grade III SCC of horn with irregular shaped tumour islands in the epidermis invading deep into dermis layer with keratin pearls (BovHC3; H& E X 100); b. tumour islands containing well differentiated epithelial pearls; Fig. 12 c. Illustrating characteristic epithelial pearl of well differentiated squamous cell carcinoma (Case ID: BovHC3; H&EX100); Fig. 12d. Large keratin cyst containing concentric cell nest (BovHC3; H&E X 100) [35]

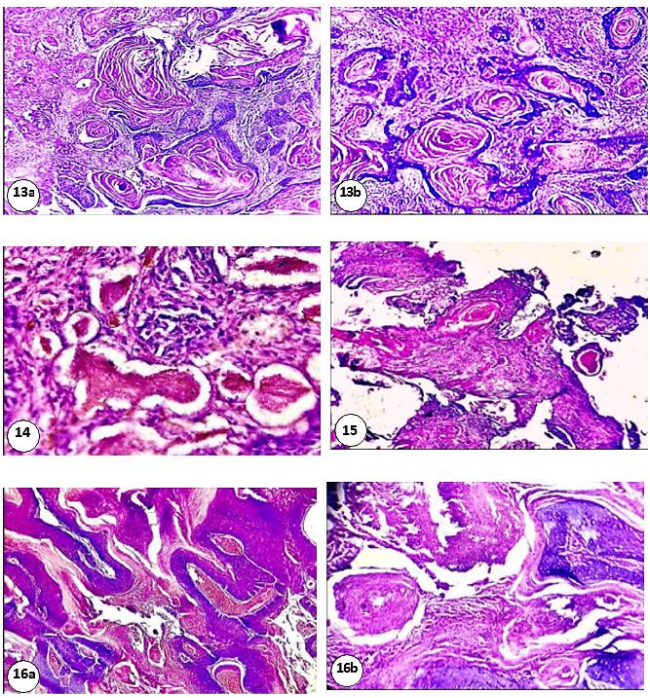

Fig. 13a. Multiple keratin pearls with formation of keratin microcyst in well differentiated SCC of horn (BovHC10; H& E X 100); Fig. 13b. concentric keratin pearls in well differentiated SCC with mineralization (BovHC10; H& E X 100); Fig. 14. Poorly differentiated SCC of horn with severe haemorrhages (BovHC4; H &E X400); Fig. 15. moderately differentiated SCC of horn with presence of epithelial pearls (BovHC8; H&E X 100); Fig. 16 a. well differentiated SCC of horn illustrating layered pattern of keratinization (BovHC9;H&E X 100); Fig. 16 b. Keratin pearls along with layered keratinization in SCC of horn (BovHC9; H&E X400)

Histopathology: Histopathological findings in SCC of horn were formation of numerous epithelial pearls with mineralization (Fig. 10a), severe keratinization of horn epithelium with concentric arrangement forming keratin pearls also called as “cell nests” (Fig. 10b). Characteristic epithelial pearl in well differentiated squamous cell carcinoma was observed (Fig. 12 c). More keratin deposition towards the center was observed in most of the cases of SCC of horn (Figs. 10a, 13a & 17a). Distinctive epithelial pearls were clearly observed in tissue samples (BovHC3; Fig. 12b, BovHC6; Fig. 17a, BovHC9; Fig. 16b). Large keratin cyst containing concentric cell nest observed (Fig. 12 d). Multiple keratin pearls with formation of keratin microcyst in well differentiated SCC of horn (BovHC10; Fig.14a) were also detected. Layered keratinization was also observed in the present study (BovHC9; Figs. 16a & b). Non keratinizing type tumours were also

seen and these cases were evident of squamous cell carcinoma (BovHC4). Tumour islands of irregular shape were clearly observed in the horn epithelium invaded deep into dermis layer (BovHC3; Fig. 13a). Numerous mitotic figures with variable number of nucleoli were observed (Figs. 17b & 19), islands of squamous epithelium with atypical squamous cells (Figs. 11b & 13a) were also found. Hyperplasia of epidermis with hyperkeratosis and pleomorphic epithelial cells arranged as cords or islands with keratinized layer in centre (accumulated in concentric manner; Figs. 11b & 13c). BovHC4 case was established as poorly differentiated SCC however deep invasion from the primary site was observed. Moderately differentiated SCC of horn was observed with presence of epithelial pearls (BovHC8; Fig. 15), large keratin pearl with numerous mitotic figures in moderately differentiated SCC (BovHC1; Figs. 11a & b). Distinctive keratin pearls were not seen in (BovHC2). Most severe haemorrhages were reported in BovHC4 (Fig. 14), severe infiltration of inflammatory cells mostly neutrophils and lymphocytes in the stroma was also observed (Fig. 17b).

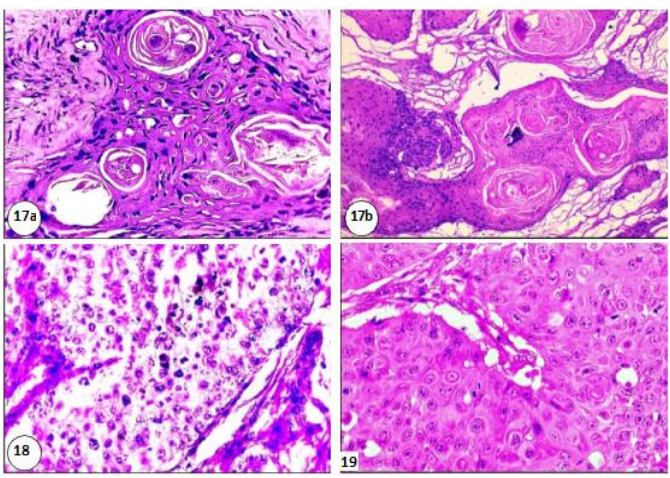

Fig. 17a. Well differentiated SCC of horn with keratin pearls as well as keratin cysts formation and numerous mitotic figures (BovHC6; H&E X 400); Fig. 17b. showing large epithelial pearls with mononuclear cells infiltration (H&E X 100; BovHC6; H&E X 100) Fig. 18. Moderately differentiated SCC with small keratin pearl (H&E X400) b. abundance of mitotic figures and cells exhibiting anaplasia Fig. 19. poorly differentiated SCC of horn with anaplastic cells and mitotic figures (BovHC4; H&E X 400) [35]

Histopathological findings of the present study were consistent with the findings of earlier workers who had reported cell nests or keratin pearls in well differentiated squamous cell carcinoma of horn [24,10,27,26,29]. Anaplasia and neovascularization observed in squamous cell carcinomas of horn were in accordance with [10,25].

Immunohistochemical study: Confirmation of horn core carcinoma was done by immunoexpression and Immunohistochemical scoring (IHS) (Table 4) Tissues were processed with 4 tumour markers (Pancytokeratin, p53, EGFR and p16) for the detection of degree of epithelial malignancy through immunoreaction of tumours to these markers.

Immunohistochemical scoring (IHS): Interpretation of results (Table 3) was done on the basis of immunoreactivity which depends on the extent of immunoreactivity and immunohistochemical staining of tumour cells. Immunohistochemical scoring of different tumour markers for SCC of horn is given in Table 4.

Table 4. Immunohistochemical scoring of different tumour markers for SCC of horn

Case ID	Hispathological diagnosis	p53 scoring	Pan-CK scoring	EGFR scoring	p16 scoring
BovHC1	Moderately differentiated SCC	0	2+	0	0
BovHC2	Poorly differentiated SCC	0	1+	0	0
BovHC3	Well differentiated SCC	4+	4+	4+	0
BovHC4	Poorly differentiated SCC	3+	3+	3+	0
BovHC6	Well differentiated SCC	2+	3+	2+	0
BovHC8	Moderately differentiated SCC	1+	1+	0	0
BovHC9	Well differentiated SCC	1+	3+	2+	0
BovHC10	Well differentiated SCC	1+	3+	1+	0

Immunohistochemical findings: SCC of horn revealed strong immunohistochemical staining of Pan-CK (Figs. 20 and 22), p53 (Figs. 30a & b), EGFR (Figs. 28, 29a and b) and negative to p16 (Figs. 33 & 34).

Immunohistochemical expression of Pancytokeratin (Pan-CK) in bovine squamous cell carcinoma: Variations observed in the immunohistochemical reactivity of Pan-CK between SCC of horn in the present study. Most of the cases of SCC of horn showed positive Pan-CK immunoreactivity in >75% neoplastic cells and was given score of **4+** (BovHC3; Fig. 20), 3+ (BovHC6; Fig. 25). Well differentiated SCC of horn showed high cytoplasmic reactivity of Pan-CK in more than 50% of neoplastic cells. Pan-CK immunoexpression was more prominent in cell nests in well differentiated SCC of horn (Fig. 21) and more intensely stained periphery/ border inside large keratin pearl (Fig. 20), well differentiated SCC of horn depicted strong cytoplasmic staining in almost all neoplastic cells, and was given the score of 4+ (Fig. 20). Reddish brown staining of Pan-CK in layered keratinization and tumour islands of BovHC9 and strong Pan-CK immunoexpression inside keratin pearl with 3+ score were also found with clear demarcation observed (Figs. 24 & 25) Strong Pan-CK immunoexpression was observed inside the cell nest with 3+ score (BovHC10; Fig. 23). BovHC6 revealed strong Pan-CK immunoexpression showing distinctive staining in keratinized portion inside cell nests (Fig. 25).

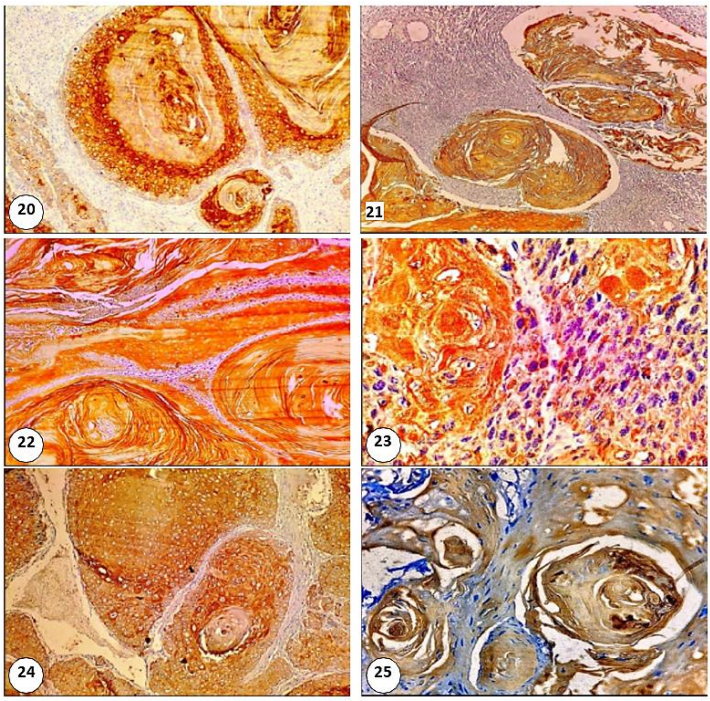

Fig. 20. Very well differentiated SCC of horn with intense Pancytokeratin immunohistochemical staining with the score of 4+ (BovHC3; IHC X 100); Fig. 21. Pancytokeratin immunoexpression in cell nests in well differentiated SCC of horn (IHC X 400). Fig. 22. Intense staining of Pan-CK inside keratin pearls leaving the peripheral region around the pearls (IHC X 400); Fig. 23. Strong Pan-CK immunoexpression inside the cell nest with 3+ score (BovHC10; IHC X 400); Fig. 24. Well differentiated SCC of horn, strong Pan-CK immunoexpression inside keratin pearl with 3+ score; BovHC9; (IHC X 100); Fig. 25. well differentiated SCC of horn, strong Pan-CK immunoexpression showing distinct staining in keratinized portion inside cell nests (BovHC6; IHC X 400) [35]

Immunohistochemical expression of EGFR in bovine squamous cell carcinoma: Variations among immunoreactivity towards EGFR were detected in the present study BovHC3 (Well differentiated SCC of horn) revealed strong immunopositive reaction against EGFR with the score of **4+** (Fig. 26). Immunoexpression of EGFR was found in the surrounding tissue excluding large keratin pearls (BovHC3; Figs. 27 & 29). BovHC4 revealed strong immunoreactivity towards EGFR with IHS of 3+ (Table 4) which signified highly malignant and invasive nature even after the absence of well differentiated epithelial pearls. mild immunexpression of EGFR was detected with 1+ score in BovHC10 (Fig. 28).

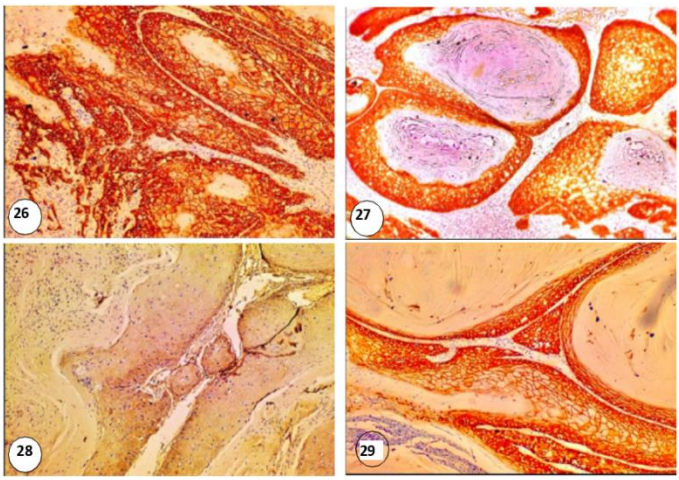

Fig. 26. EGFR immunoexpression with intense staining of tumour islands showing score of 4+ (BovHC3; IHC X 100) Fig. 27. EGFR immunohistochemical staining in periphery of keratin pearls with absence of immunoexpression inside cell nests (BovHC3; IHC X400); Fig. 28. Mild immunoexpression of EGFR with 1+ score (BovHC10; IHC X 400) Fig. 29. EGFR immunoexpression in the surrounding tissue excluding large keratin pearls (BovHC3; IHC X 400)

Immunohistochemical expression of p53 gene in bovine squamous cell carcinoma: In the present study, Immunohistochemical reactivity scoring of p53 is based on number of positive tumour cells and intensity of staining of nuclei of tumour cells. Pattern of staining varied from moderate to intense. The concentration of p53 gene increased in response to the DNA damage inside the nucleus of tumourous cells and was responsible for its immunoexpression. Immunoexpression of p53 in tumour nuclei was more pronounced in well differentiated SCC of horn (Fig. 31b). SCC of horn exhibited p53 nuclear staining of tumour cells with 4+ score (BovHC3; Figs. 30a & b), scattered in connective tissue stroma and peripheral neoplastic cells of tumour islands (Fig. 31a) showing intense nuclear staining of p53 with **4+** score (Fig. 30b). One case of horn cancer (poorly differentiated) revealed high staining in more than 50% neoplastic cells in tumour islands (Figs. 31a & b). Moderate immunoexpression of p53 in nuclei of tumour cells with 2+ score observed in BovHC6 (Figs. 32a & b) and more immunoexpression was detected within the outer epithelial layer of keratin pearl (Fig. 32a).

Immunohistochemical expression of p16 in bovine squamous cell carcinoma: Samples showed negative reaction/ non-immunoreactive towards p16 (Figs. 33 & 34) including both well differentiated (Figs. 33a & b) and poorly differentiated SCC (BovHC4; Figs. 34 & 35).

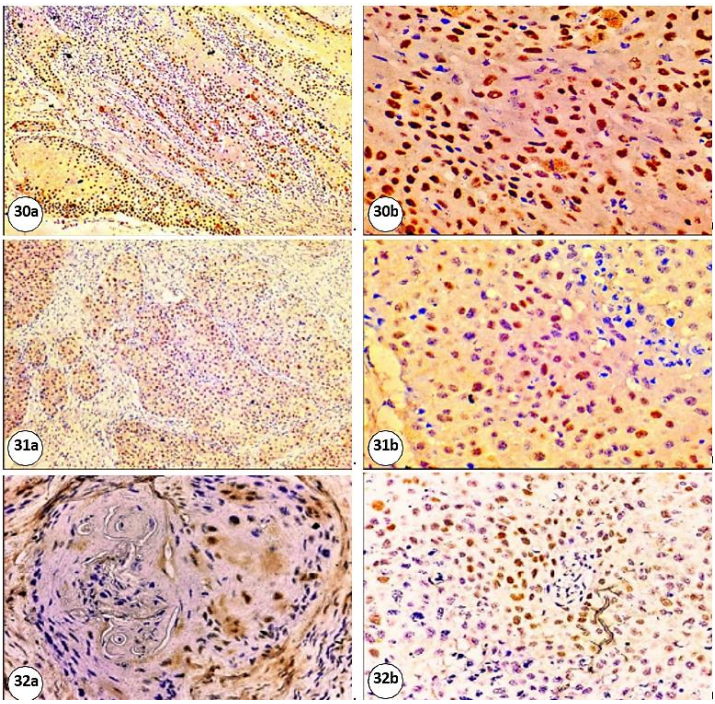

Fig. 30a. well differentiated SCC of horn, p53 nuclear staining of tumour cells with 4+ score (BovHC3; IHC X 40); Fig. 30b Higher magnification of fig. no. 30a. showing intense nuclear staining of p53 with 4+ score (BovHC3; IHC X400); Fig. 31a. poorly differentiated SCC of horn, p53 Immunohistochemical expression in BovHC4 with 3+ score (IHC X 100); Fig. 31b) Higher magnification of fig. no. 37a. (BovHC4; IHC X 400) Fig. 32 a. Moderate immunoexpression of p53 in nuclei of tumour cells with 2+ score (BovHC6; IHC X 400); Fig. 32b. p53 immunoexpression in BovHC6 with 2+ score (different field; IHC X 400)

Immunohistochemical findings of the present study were partially in accordance with the findings of Sharma et al. [10]. They depicted strong cytoplasmic staining in almost all neoplastic cells in poorly differentiated SCC of horn and another case of poorly differentiated SCC of horn which showed moderate staining in about 75% of neoplastic cells, but present study revealed highest immunoexpression and intense staining of Pan-CK in well differentiated SCC of horn (BovHC3) although high and moderate immunoexpression of Pan-CK was observed in poorly and moderately differentiated SCC are in accordance with Kumar et al. [12]. p53 immunoexpression in nuclei of tumour cells around periphery of keratin pearls, sparing the region of keratinization with p53 tumour marker was also reported by Carvalho et al. [30], Fornazari et al. [21] and Sharma et al. [10] High immunoreactivity observed in well differentiated SCC of

horn in the present study differed from the findings of Sharma et al.]10] which stated strong immunopositive reaction of p53 in poorly differentiated SCC of horn. Fornazari et al. [21] observed intense positive immunostaining of p53 and expression mostly within outer epithelial layer of the cell nests. The concentration of p53 increases in response to DNA damage in the nucleus of the cells and also inactivation of tumour suppressor (p53 gene) is the possible mechanism for oncogenesis. The present findings of immunoexpression of EGFR were in accordance with Lakshmi et al. [21]. They observed strong immunopositive reaction against EGFR in OSCC. High immunopositive reaction of EGFR confirmed the malignant tendencies of tumour of epithelial origin. Higher activity of EGFR signifies the increased growth, invasiveness and metastasis of squamous cell carcinoma [16]. Most of the tissue samples showed negative reaction/ non-immunoreactive towards p16 gene. Pattern of immunohistochemical staining of tumour markers varied in SCC of horn. Strongest immunoreactivity was observed for pan-cytokeratin marker. SCC of horn exhibited strong staining in more than 50% of neoplastic cells for all 3 markers (Pan-CK, p53 and EGFR) [31-34].

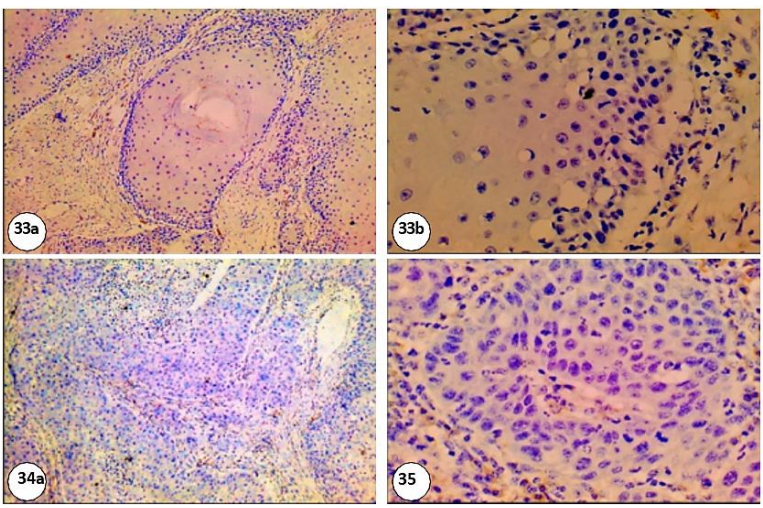

Fig. 33 a. well differentiated SCC of horn, negative immunoreaction of p16 (BovHC3; IHC X 100); Fig. 33b) Higher magnification of fig. no.33a (IHC X 400); Fig. 34. poorly differentiated SCC of horn, negative immunoexpression of p16 with IHS 0 (BovHC4; IHC X 100); Fig. 35. Negative immuhistochemical expression of p16 (BovHC10; IHC X 400).

Grading of SCC (horn) on basis of both histopathology (HP) and immunohistochemistry (IHC) revealed a total of two cases as Grade I (poorly differentiated SCC); 2 cases as Grade II (moderately differentiated SCC) and four cases as Grade III (well differentiated SCC) (Table 5).

Table 5. Grading of SCC on the basis of HP & IHC

Grade of malignancy	SCC of horn
Grade I (Poorly differentiated SCC)	2
Grade II (Moderately differentiated SCC)	2
Grade III (Well differentiated SCC)	4
Total	8

4. CONCLUSION

A total of Eight out of Twelve tissue samples collected were confirmed as horn core carcinoma on the basis of histopathology and Immunohistochemistry. Cytological examination revealed variation in shape and size of cells, altered nuclear details; tumour cells were observed with cells stained more intensely with hyperchromasia and anaplasia. Unilateral growth was observed in all the cases examined, grossly tumours suspected of SCC were large cauliflower like ulcerated growth with rough and verrucous surface. Histopathologicaly, cell nests or keratin pearls with high degree of keratinization and layered pattern of keratinization were reported in well differentiated SCC of horn with anaplasia, numerous mitotic figures, tumour islands with severe inflammation, neovascularization, haemorrhages etc. SCC of horn revealed strong immunohistochemical staining of Pan-CK, p53, EGFR and negative to p16. High Pan-Cytokeratin immunoreactivity confirmed the tumours of epithelial origin and EGFR immunoexpression was confirmatory for malignancy and degree of metastasis [35].

CONSENT FOR PUBLICATION

All the authors consent to the publication of this manuscript.

CONSENT AND ETHICS APPROVAL

This research followed the guidelines specified by Institutional Animal Ethics Committee (IAEC). All the experimental procedures were carried out as per recommendations of the IAEC.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 7
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Exploring Ocular Morphological Alterations in Iron Deficiency Anemia through Optical Coherence Tomography: A Comprehensive Analysis

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ABSTRACT

Anemia, characterized by insufficient red blood cells and impaired oxygen-carrying capacity, affects billions globally, with a high prevalence among women and children. This study explores the multifactorial etiology of anemia, focusing on preventable factors such as inadequate diet, poor living conditions, and infections. Optical Coherence Tomography (OCT), a diagnostic tool offering high-resolution cross-sectional imaging of biological tissues, is employed to investigate the ocular impact of iron deficiency anemia (IDA). Various studies utilizing OCT reveal consistent correlations between IDA and structural changes in the peripapillary retinal fiber layer (RNFL) and macula. These changes are associated with serum ferritin levels, hemoglobin concentrations, and other iron profile parameters. In conclusion, this comprehensive review and study underscore the significance of OCT in evaluating ocular changes associated with iron deficiency anemia. The consistent patterns observed across multiple studies emphasize the potential clinical utility of OCT as a non-invasive tool for monitoring and managing IDA-related ocular manifestations. This research lays the foundation for further exploring and applying OCT in clinical assessments,

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offering valuable insights for early detection and intervention in preserving ocular health in anemic populations.

Keywords: Optical coherence tomography; red blood cells; iron-deficiency anemia, malaria.

1. INTRODUCTION

Anemia is a condition in which the number of red blood cells (RBCs) and their oxygen-carrying capacity are insufficient to meet the body's physiologic needs [1]. It affects an estimated 2.36 billion individuals globally, especially women and children [2]. According to the National Family Health Survey 4 (NFHS-4), 58.4% of children aged 6–59 months, 53.1% of non-pregnant women aged 15–49 years, 50.3% of pregnant women aged 15–49 years, 53% of all women aged 15–49 years, 22.7% of men aged 15–49 years, 54% of adolescent girls and 29% of adolescent boys were anemic in India [3].

The etiology of iron- deficiency anemia is multifactorial, but for the most part, the etiology of iron-deficiency anemia is preventable and includes inadequate diet, poor living conditions, and high infection rates (i.e, malaria and intestinal parasites), etc [4].

2. OPTICAL COHERENCE TOMOGRAPHY (OCT)

Optical coherence tomography is a diagnostic tool that can perform cross-sectional or tomographic images of biological tissues within less than 10 µm axial resolution using light waves. The operation of OCT is analogous to Ultrasonography-B (USG-B) imaging [5,6].

OCT Imaging: The internal cross-sectional microstructure of tissues using measurements of optical backscattering or back reflection, was first demonstrated in [7]. OCT imaging was performed in vitro in the human retina and atherosclerotic plaque as examples of imaging in transparent, weakly scattering media and nontransparent, highly scattering media. OCT was initially applied for imaging in the eye, and to date, it has had the most significant clinical impact in ophthalmology [8]. OCT imaging was expanded upon with the development of OCTA which uses motion contrast to identify blood flow. When two consecutive photos of a scene are captured, immobile things remain unchanged, but moving objects will be visible. OCTA records consecutive A-scans at the identical retinal position, with each scan being separated by a short interval of time. When light is reflected, a discrepancy in signal will be observed in the two scans. The variation is caused by movement between the scans and is referred to as decorrelation signal. The decorrelation signal in the retina is caused by the movement of blood through the retinal vasculature, as the retina itself is a static tissue [9]. Enhanced depth imaging in spectral-domain optical coherence tomography (EDI SD-OCT) utilises a closer scanning proximity to the eye to generate an inverted SD-OCT image that offers improved depth sensitivity. This enables precise observation of choroidal anatomy and accurate measurement of choroidal thickness (CT). Currently, there is an increasing number of choroidal thickness measurements

being conducted in both normal and pathological conditions to better comprehend the development and differentiation of various diseases, particularly macular lesions [10].

3. OPTICAL COHERENCE TOMOGRAPHY IN IRON DEFICIENCY ANAEMIA

Iron is essential in oxygen transport, normal myelination, neurotransmitter synthesis, and neurometabolism [11]. In retinal tissue, iron is vital for the visual photo-transduction cascade. Iron is an essential cofactor for the enzyme guanylate-cyclase, which synthesizes cGMP, the second messenger in the phototransduction cascade. Iron may play a role in ocular diseases, including glaucoma, cataracts, AMD (age-related macular degeneration), and conditions causing intra-ocular hemorrhage [12,13].

Peripapillary RNFL thickness is found to be associated with serum ferritin levels [14]. A positive correlation between mean RNFL thickness and hemoglobin, ferritin, and transferrin saturations while a negative correlation was also found between total iron binding capacity and mean RNFL thickness [15]. In pediatric population with IDA thinner peripapillary RNFL profiles were recorded with a statistically significant correlation in all quadrants [16].

Evaluation of retinal vascular parameters using optical coherence tomography angiography in patients with iron deficiency anemia (IDA) and healthy children (control group) had significantly lower capillary plexus density as reported with the retinal microvascular changes, especially ischemic changes, in young IDA patients before the development of significant ocular anomalies [17]. The first study of retinal vessel OCT images for anemia screening, indicated that OCT-A can be used to investigate the effect of iron deficiency anemia (IDA) on macular and radial peripapillary capillary (RPC) vascular changes [18]. A positive association between average, nasal, and inferior RNFLT and Hb concentration was also eva;uated by researchers in both eyes and the degree of thinning was related with the severity of anemia [19].

A significant decrease in vessel thickness of the superficial (SCP) and deep capillary plexus (DCP) compared to healthy was concluded in patients with IDA in parafoveal vessel density in the SCP, and foveal avascular zone metrics and choriocapillaris flow area measurements [20].

In the patients with IDA in Eastern India, decreased mean peripapillary RNFL thickness, and a strong positive correlation was noted between RNFL thickness and Hb and other iron profile parameters excluding TIBC [21]. Enhanced-depth imaging optical coherence tomography (EDI- OCT) was used to measure the macular and choroidal thicknesses in the temporal and nasal subfoveal areas and it was seen that patients with iron-deficiency anemia had a significantly reduced choroidal thickness [22].

A statistically significant reduction of the thickness of peripapillary RNFL thickness measured by OCT in adult female patients with IDA compared to healthy controls was suggestive of its role in glaucoma and neuro-ophthalmological disorder, along with early evaluation of IDA [23]. It was also found significnt in thalassemia, anemia of chronic disease, and IDA patients [24]. RNFL was significantly thinner in all quadrants in the thalassemia major group vs. the other two groups and only the inferior quadrant in the IDA group [25].

The mean RNFL thicknesses in OCT in all quadrants, i.e, Superior, Inferior, Nasal, and Temporal quadrants, and average values in the IDA group were thinner as compared to the control group and were statistically significant in both eyes in a study conducted by the authors. Similarly, the central macular thickness was significantly lighter in the iron deficiency anemia group than in the control group.

A significant decrease in mean RNFL thickness of both eyes in the Superior, Inferior, Nasal, and Temporal quadrants, and the Average value in case subjects was lower than that of the age and sex-matched controls [15,26]. RNFL thicknesses of inferior, superior, and temporal quadrants was also recorded in pediatric IDA patients which was statistically significant [16].

A positive association between average, nasal, and inferior RNFL thickness and Hb concentration in both eyes and a degree of thinning related to the severity of anemia was found in researh studies [19,21].

The patients with iron-deficiency anemia had a significantly reduced choroidal thickness in both the genders. It was suggested that OCT may significantly impact the assessment of many diseases, such as glaucoma and neuro-ophthalmological disorder, along with early evaluation of IDA [21,22]. Correaltion of RNFL changes with the hemoglobin level and a strong correlation of RNFL thinning with a degree of anemia in all quadrants in pediatric and young adult population both has been found in iron deficiency anemic case subjects [26,27].

The mean Central Macular Thickness in the left eye in the subjects was reported to be less than in the control population, and similar findings were reported in the right eye. Both the findings were statistically significant in a unpublished post graduate approved academic study by the authors. Iron affects myelin synthesis directly as a cofactor of cholesterol and lipid biosynthesis and indirectly as an oxidative metabolism component in oligodendrocytes. Demyelination of nerve fibers results in the decreased thickness of the RNFL [28,29]. Deficiency of retinal dopaminergic dysfunction is thought to alter the receptive area of axons and ganglion cells, which constitute the retinal nerve fiber layer (RNFL) [12,30].

The correlation coefficient between OCT and the anemia profile was calculated using Pearson's correlation by the authors in their study; a moderate positive correlation between Hb and CMT in controls and Transferrin saturation and CMT was calculated in cases in the right eye. The controls calculated a moderate negative correlation between Hb and nasal RNFL and CMT in the left eye.

Similarly, mild correlations, positive and negative, were seen in both eyes between the OCT and Iron study parameters. It has been determined that factors such as vasospasm, venous stasis, and hypoxia are essential in forming anemic retinal nerve fiber and choroidal disorders [15,17]. Retinal ganglion cell and nerve fiber layer development may be affected in patients with IDA [15,17,31].

As not many studies are reported in the literature including both these parameters, OCT evaluates and detects the early changes in RNFL thickness in patients of IDA and can act as the prognostic imaging tool for IDA patients and in follow-up patients of IDA on treatment [18].

4. CONCLUSION

In conclusion, the comprehensive analysis of relevant literature and the findings of the author's study underscore the significance of OCT in assessing the ocular manifestations of iron deficiency anemia. The identified correlations between OCT parameters and anemia profiles contribute valuable insights into the potential use of OCT as a non-invasive tool for monitoring and managing IDA-related ocular changes. This research opens avenues for further exploration and application of OCT in the clinical assessment of anemic patients, highlighting the importance of early detection and intervention in preserving ocular health in this population.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Nutritional Aspects of Various Edible Crustaceans in India

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ABSTRACT

The present study highlights about stimulate interest in the fascinating group of organisms by summarizing what little we do know about lipids and fatty acids of freshwater crabs in India. We explore carbohydrate, protein and lipid contents of an animal because of utilization of those components for our betterment i.e. resource utilization for health. Thus, human exploit and incorporate many such animals in their food chain. Lipids play important roles in the metabolism and reproduction of decapods crustaceans. Indian prospect is no better where epidemic of coronary heart disease (CHD) and diabetes is ongoing with no signs of a recession. Edible crustaceans, including prawns, crab, shrimp, crayfish, and lobster, are a key source of nutrient-dense food for humans. The biochemical makeup of crustaceans, including their protein, amino acid, lipid, fatty acid, carbohydrate, vitamin, and mineral content, determines their nutritional value. Freshwater crabs are a cheap food source that have a lot of potential for human consumption in India, where they can be found in almost all freshwater habitats. However, basic information about these creatures, like the biochemical makeup of their body flesh and hepatopancreas, which humans can consume, is still unknown and has not been linked to any specific nutritional benefits for the Indian populace. Freshwater crabs are neglected in various parts of world as well as in India also. But they have good quality proteins and fatty acids in their edible part. In this article, occurrence of major lipid and fatty acids were addressed. Among fatty acids, SFA especially C16 total and C18 total; MUFA, PUFA, linoleic acid (18:2ω6), linolenic acid (18:3ω3), EPA (20:5ω3) and DHA (22:6ω3) were compared in body flesh of various edible crustaceans. It was observed that Indian freshwater crab have a high content of MUFA and PUFA which makes them nutritionally attractive. The fatty acid profile exhibit a greater percentage of EPA and DHA of total fatty acids. The n3/n6 ratio maintains less than 1% in the body flesh of crabs. Exploration of the nutritional quality of freshwater crab will encourage farmers to promote freshwater crab culture in

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India. As well as the consumption of freshwater crab may help to prevent nutritional deficiencies in future and the topic is important for that.

Keywords: Crustacean; CVD; freshwater crab; lipid; fatty acid; n3/n6 ratio.

1. INTRODUCTION

There are several animal specially crustaceans, majorly crabs uptake all sorts of nutrients through their food source not for our consumption but for their own physiological requirement. Crab meat is reported as providing nourishment to the human body thus increases cognition, reduces inflammation, strengthens the bones, boosts the immune system, stimulates blood circulation and detoxifies the body. In general, crab meat contains appreciable quantities of digestible proteins, essential amino acids, bioactive peptides, long-chain polyunsaturated fatty acids, astaxanthin and other carotenoids, vitamins especially vitamin B12, minerals, including copper, zinc, inorganic phosphate, sodium, potassium, iron, iodine, and also other nutrients, which offer a variety of health benefits to the consumer [1]. We explore carbohydrate, protein and lipid contents of an animal because of utilization of those components for our betterment i.e. resource utilization for health. Thus, human exploit and incorporate many such animals in their food chain.

The population of India constitutes more than one third of the developing world and among them more than half live below poverty level, can't be called a health conscious group. The common Indian seems to be aware of their food for keep them fit and well to work hard. They are very familiar with the protein for make them wealthy but fat or lipid or fatty acids seem to be technical and usually avoided by them in food and nutrition. People are more concerned these days about the intake of protein value and diet chart contains less fat for less calorie ingestion. Health conscious public recognizes the importance of lipid as a vital dietary component because of the role of lipid and fatty acid content in the diet for cardiovascular impairment. A high caloric diet and least physical activity contribute to the modern day health problems like dyslipidemia, obesity, etc. All of these increase the risk of cardiovascular disease (CVD). Indian prospect is no better where epidemic of coronary heart disease (CAD) and diabetes is ongoing with no signs of a recession [2]. According to them, Indians should increase the MUFA intake to 20% with the total fat intake to 35% of the energy because of the beneficial effects on High Density Lipid (HDL) and Triacylglycerols (TAG). Edible crustaceans, such as crab, shrimp, prawn, crayfish and lobster constitute one of the major sources of nutritious food for human being. Among seafood, prawns and shrimps contribute about 20% by volume of the world seafood market. Seafood in general, prawns and shrimps in particular, are highly nutritious with good source of protein and amino acids.

The nutritive values of crustaceans depend upon their biochemical composition, such as protein, amino acids, lipid, fatty acids, carbohydrate, vitamins and minerals. Being very cheap freshwater crabs constitute a great food potential for

human in India. A large part of these shell fish species from cultivated farms. So there is growing need for information about the biochemical composition of these shell fishes. For the cultivation of these shellfishes some important characteristics, such as nutritional properties, biochemical structure and growth conditions need to be identified. Human consumption of freshwater crabs has been recorded from various parts of Africa, including *Sudanonautes aubryi* in Ivory Coast [3] and *S. africanus* and *S. kagoroensis* in Nigeria [4,5]. In Liberia, the dwarf river crab *Liberonautes nanoides* is caught in large numbers during the dry season using basket traps, and sold in local markets for human consumption (Sachs and Cumberlidge, 1991). In Nigeria, *Sudanonautes africanus* is commonly sold in markets and roadside stalls, either fresh or smoked [4]. Crab consumption in this region tends to increase when economic decline reduces the availability of other protein sources [6]; economic austerity will therefore lead to increased prevalence of paragonimiasis among susceptible human populations. Recently in India few works were done on freshwater crabs. Radhakrishnan and Natrajan [7] investigated the lipid content in *Podopthalmus vigil* (Fabricius); Manhas *et al*. [8] studied water and lipid distribution pattern in female *Paratelphusa masoniana* (Henderson), an edible freshwater crab from Jammu region of J&K; Jadav (2013) observed, impact of mercuric chloride on lipid in the freshwater crab, *Barytelphusa guerini*; Das *et al*. [9] reported major lipid classes and their fatty acids in the flesh and hepatopancreas of an edible freshwater crab *Varuna litterata* (Fabricius 1798). However, freshwater crabs are strangely neglected component of the world's inland aquatic ecosystems. Despite their wide distribution throughout the tropical and warm temperate zones of the world, and their great diversity, their role in the ecology of freshwater is very poorly understood. Similar situation prevails in India, where crabs occur in almost every freshwater habitat, yet even fundamentals such as their biochemical composition of body flesh and hepatopancreas (these two parts are normally consumed by human) are yet to be determined and correlated with nutritional aspects for Indian population. This review will attempt to stimulate interest in this fascinating group of organisms by summarizing what little we do know about lipids and fatty acids of freshwater crabs in India.

2. LIPIDS

Crustaceans use lipid for numerous biological structures and processes [10]. The most studied decapod crustaceans in terms of lipid concentrations and ovarian maturation are penaeid shrimp [11-16]. Lipids play important role during the development of decapods crustaceans, not only as energy source, but also as essential nutrients [17]. In crustaceans, the hepatopancreas is generally regarded as a major lipid storage organ. In the case of female crustaceans, ovaries also contain higher levels of lipid than other organs and this suggests that lipids are important for maturation of ovaries [18,19], as well as precursor of gonadal steroids. The hepatopancreas is the main lipid storage organ, triglycerides and phospholipids being its major lipid components, while the muscle contained mainly phospholipids [20,21,9]. Therefore, prawns and other sea foods are preferred by the consuming communities. Lipids also form a major component of yolk in decapod crustaceans. The majority of lipids stored in

oocytes are derived from extraneous sources, particularly the hepatopancreas [22]. Lipids are the precursors of steroidal hormones. The higher quantity of total lipid and fatty acids recorded in the adult female prawns may be necessitated for performing certain specific physiological activity related to reproduction. It has been reported that incorporation of essential fatty acids in the diet produced better growth rate and survival in aquaculture [23,24,25]. Lipids are extremely important in maintaining structural and physiological integrity of cellular and sub-cellular membranes. Lipids are the best source of energy producers of the body through metabolism. They provides a source of indispensable nutrients and act as carriers of certain non fat nutrients, notably the fat soluble vitamins like A, D, E and K [26,27]. The proximate body composition including moisture, fat, protein and ash are good indicators of physiological condition of an organism. The greater the protein and lipid content represents higher the energy density [28]. However, quantities of these constituents vary considerably within and between species, size, sexual condition, feeding season and physical activity [29,30].

3. FATTY ACIDS

Lipids and fatty acids play important roles in the biochemistry, metabolism and reproduction of decapod crustaceans. Neutral lipids, particularly triacylglycerols, are a major energy source, and the predominant form of energy storage in the adult, egg and pre feeding larvae [31, 19 and 32]. Phospholipids and sterols have important function as cytoplasm and membrane constituent of cells, affecting structural and physiological properties. Polyunsaturated Fatty Acids (PUFA) are important component of lipids and are essential for marine fish and crustaceans [33]. Apart from being a major role of metabolic energy and main form of energy storage, lipids also supply essential fatty acids needed for the maintenance and integrity of cellular membranes and serve as precursor of steroid and molting hormones [12,34]. Lipids play significant role during gonadial growth, maturation and development of decapod crustaceans. They are very important food reserves in the oocytes [35,36]. The high lipid content observed in spring and post-monsoon could be attributed to active feeding and optimum availability of food, as algal blooms and plankton are reported to acquire maxima during this period [37]. It is well known that marine animals generally contain large amounts of polyunsaturated fatty acids with a long carbon chain, whereas terrestrial animals involve relatively large amounts of saturated C16 and C18 acids. As to the crustaceans, many reports have been presented about the fatty acid composition of lipids from different parts of world; for example, mysids, *Neomysis interger* [38]; *Jasus lalandii* [39]; shrimps, *Pandalus borealis* [40]; *Homarus americanus* [4]; *Euphausia* sp. [42, 43] euphausids, *Meganyctiphanes* norvegica [40] and copepods [44, 45)]; prawn, crabs, *Pleuroncodes planipes* [46, 47] *Euphausia superba* (Hansen, 1969); *Thysanoessa inermis* [48]; *Cancer magister* [10]; *Xiphosura* (Limulus) *polyphemus* [49]; *Crangon septemspinosus* [50]; lobsters, *Penaeus japonicas* [51].

The higher levels of EPA and DHA would increase stress tolerance and membrane permeability [52,53]. The arachidonic acid (n-6) is a precursor of prostaglandin hormone, which is essential for reproduction and vitellogenesis

[54,55,25]. The interaction and balance between ω-3, ω-6 and ω-9 fatty acids are crucial for maintenance of good health [56,57]. The ω-3 fatty acids have anti-inflammatory and anti-coagulant properties as well as many other important health benefits. The DHA is important for pregnant and nursing mothers and in young children for healthy development of the brain and vision. The EPA can be considered the most important for everyone else as it is necessary for continuation of the efficient functioning of the brain and body at the cellular level. The ω-6 fatty acids have their own role in female reproductive cycle. The ω-9 fatty acids help to reduce the risk of arteriosclerosis, cardiovascular disease and stroke. In Egypt, Flower [58] reported that crabs were actively sought and eaten by childless women, in the belief that this would cause them to become pregnant. In India Das *et al.* [9] extensively investigated the detail lipid components and their fatty acids from the flesh and hepatopancreas of a freshwater crab, *Varuna litterata,* and suggested that this freshwater crab have good source of lipids and fatty acids and this species can be used as marine counterpart. Since freshwater crabs containing considerable amounts of PUFA it can provide a healthy choice of daily diet.

4. OTHER NUTRITIONAL ASPECTS

Crustacean muscles also contain high concentration of free amino acids, such as arginine, glycine, proline, glutamine and alanine [59]. The free amino acids have been shown to function in osmoregulation [60] and have major contribution to the flavor of sea food [61]. The amino acid, tryptophan plays an important role in the brain as a precursor of the neurotransmitter, serotonin, which has a major effect on the feeding behavior of animals [62]. Valine is involved in many metabolic pathways and is considered indispensable for protein synthesis and optimal growth [63]. Histidine is also an indispensable amino acid involved in many metabolic functions including the production of histamines, which take part in allergic and inflammatory reactions. It plays a very important role in maintaining the osmoregulatory process and is related to energy production or is used in other metabolic pathways during certain emergencies/ harsh conditions (Abe and Ohmama, 1987).

Crabs are put to various medicinal uses. One of the most interesting is the role of *Potamonautes raybouldi,* the tree hole crab of the East Usambara Mountains in Tanzania and the Shimba Hills in Kenya [64,65]. Here it is not the crab itself that is important, but the water from the tree hole in which it lives. Tree hole crab water is administered to pregnant women, and particularly those with a history of miscarriages. The value of this water may relate to the behavior of the crab, which neutralizes the naturally acidic water in tree holes by capturing snails and adding their crushed shells to the water, raising the pH but also enhancing levels of dissolved calcium [64].

Crabs may play a valuable role as indicators of pollution. *Potamonautes warreni* has the misfortune of being large-bodied, easy to capture with bait, and common in the Orange River, which drains much of the heavily polluted mining region of

northern South Africa. Therefore it has been intensively investigated as a possible bioindicator of metals in sediments (e.g.66,67,68]. One species, *Potamonautes lirrangensis* ('Malawi blue crab'), which occurs in Lake Malawi and in rivers in the upper Congo catchment, can be found for sale as an aquarium species (often under the name of P. *orbitospinus*). The consumption of freshwater crab may help to prevent nutritional deficiencies in future and the paper may be have sufficient information.

In this article, total lipid content of some edible freshwater and marine water crustacean is to be observed (Table 1). The content of total lipids is high in marine crustacean relatively to freshwater crabs. In most cases freshwater crabs shows less than 2% fat which makes them lean and good for cardiac patients. Table 2 provides SFA components of different edible crustaceans. It is seen that freshwater crab *V. litterata* has high SFA content followed by *S. dehaani*, a brackish water crab. Fig. 2 shows a comparison in C-16total and C-18total of different edible crustaceans. The MUFA and PUFA components are also observed and Table 3 and Table 4 presents profiles of edible crustacean's fatty acids. Both UFAs are dominated in marine crustaceans but freshwater individuals have remarkable presence of EPA and DHA (Fig. 3), which makes them nutritionally attractive. The n-3/n-6 ratio in freshwater crustaceans (Table 5) mostly has little value when compared to that of marine or brackish water crustaceans.

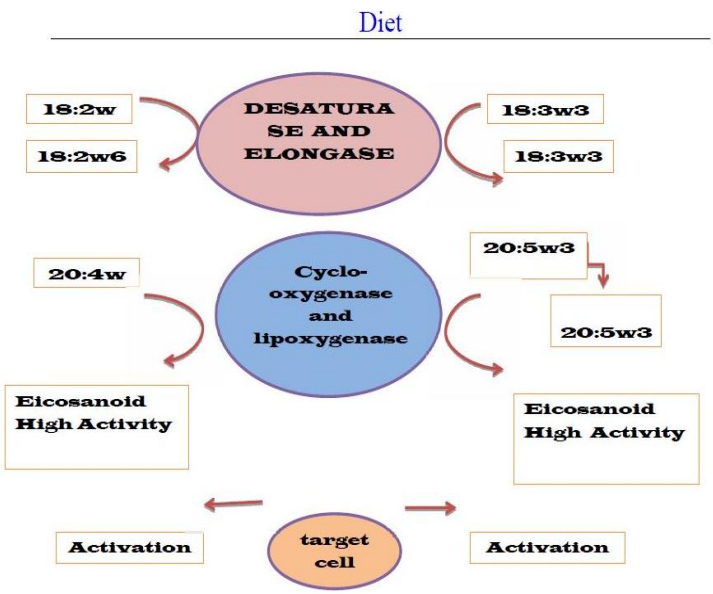

Fig. 1. Relative links between dietary PUFA, tissue PUFA and eicosanoid product (modified after [94])

Table 1. Comparative studies of total lipid content in different edible crustaceans (from available sources)

Edible crustacean	TL%	References
Cray fish (From semi finished canned products)	0.4-0.9%	Walkowiak,[69]
P. vigil	16.8-31.9%	Radhakrishnan ,[7]
I. crenata	5.4-15.6%	Thomas ,[70]
M. rosenbergii	3.37%	Gopakumar, [71]
S. tranquebarica	1.8-2.7%	Gopakumar, [71]
S. serrata	0.21%	Gopakumar, [71]
Cray fish (from different habitats)	0.15-0.3-%	Wlasow, [72]
Cray fish (from polish waters,)	0.15-0.3%	Wlasow, [72]
M. rosenbergii (male)	3.35-5.35%	Bhavan *et al*., [74]
M. rosenbergii (female)	4.12-6.34%	Bhavan *et al*., [74]
Cray fish (Goplo lake, Poland)	0.92-1.10%	Stanek *et al*., [75]
P. mansonia	5.85%	Manhas *et al*., [8]
V. litterata	1.03%	Das *et al*., [9]
P.lamellifrons	17.64%	Islam *et al.,*[76]

Table 2. SFA components of different edible crustaceans (from available sources)

Edible crustacean	SFA%	References
P. japonicus	26.90%	Teshima *et al.,*[19]
H. tridens	28.00%	Teshima *et al.,*[19]
S. dehaani	35.10%	Teshima *et al.,*[19]
P. paucidens	17.40%	Teshima *et al.,*[19]
S. serrata	23.25%	Anas *et al*., 2009
O. limosus (Brda river, Poland)	21.26%	Stanek *et al.,* [77]
O. limosus (Goplo lake, Poland)	21.97%	Stanek *et al.,* [75]
V. litterata	41.10%	Das *et al*., [9]
P.lamellifrons	25.96%	Islam *et al.,*[76]

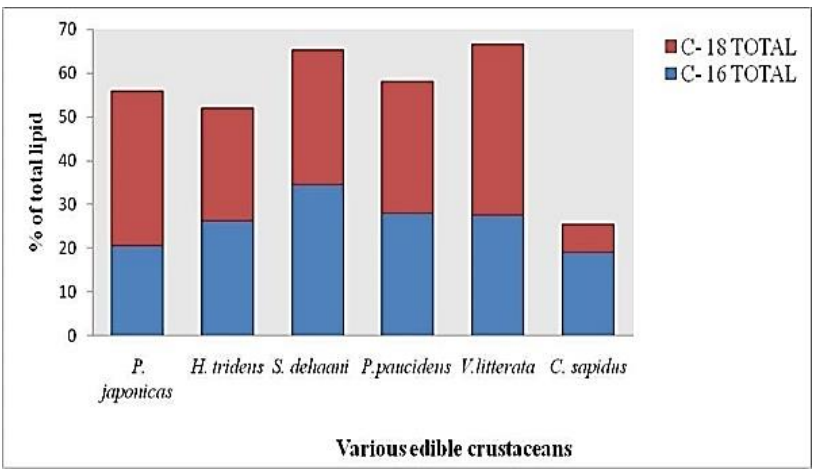

Fig. 2. Comparison of total C16 and total C18 of different edible crustaceans

Table 3. MUFA components of different edible crustaceans (from available sources)

Edible crustacean	MUFA%	References
P. japonicus	25.10%	Teshima *et al.,*[19]
H. tridens	27.80%	Teshima *et al.,*[19]
S. dehaani	38.60%	Teshima *et al.,*[19]
P. paucidens	40.50%	Teshima *et al.,*[19]
S. serrata	25.80%	Anas *et al*., 2009
O. limosus (Brda river, Poland)	29.05%	Stanek *et al.,* [77]
O. limosus (Goplo lake, Poland)	30.36%	Stanek *et al.,* [75]
V. litterata	29.70%	Das *et al*., [9]
P.lamellifrons	42.85%	Islam *et al.,*[76]

Table 4. PUFA components of different edible crustaceans (from available sources)

Edible crustacean	PUFA%	References
P. japonicus	47.90%	Teshima *et al.,*[19]
H. tridens	44.10%	Teshima *et al.,*[19]
S. dehaani	26.30%	Teshima *et al.,*[19]
P. paucidens	42.00%	Teshima *et al.,*[19]
Cray fish (From semi finished canned product)	34.70%	Walkowiak,[69]
S. serrata	42.85%	Anas *et al*., 2009
O. limosus (Brda river, Poland)	39.18%	Stanek *et al.,* [77]
*O. limosus (*Goplo lake, Poland)	48.38%	Stanek *et al.,* [75]
V. litterata	19.05%	Das *et al*., [9]
P.lamellifrons	15.02%	Islam *et al.,*[76]

Table 5. The n3/n6 ratio of different edible crustaceans (from available sources)

Edible crustacean	n-3/n-6 ratio	References
H. gammarus (female)	4.20%	Barrento *et al.,* [78]
C. pagurus	3.50%	Barrento *et al.,* [79]
O. limosus (Brda river, Poland)	0.72%	Stanek *et al.,* [77]
O. limosus (Goplo lake)	0.70%	Stanek *et al.,* [75]
V. litterata	1.58%	Das *et al*., [9]

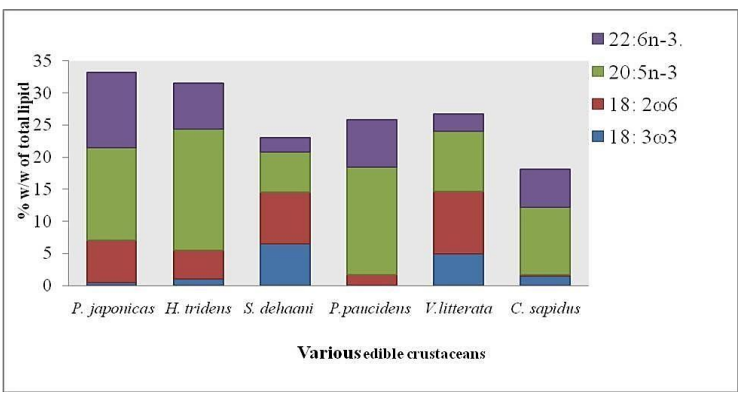

Fig. 3. Comparisons of four valuable fatty acids for human in different edible crustaceans. The prawn, *P. japonicus*, was obtained from Darumaya-Sangyo Co., Kagoshima. The crabs, *H. tridens tridens* and *S. dehaani*, were collected in the marsh quite near brackish water at Ibusuki, Kagoshima; The crab, *H. tridens tridens*, was inhabiting in the wet mud of relatively shallow depths, whereas *S. dehaani* was found in the hole of relatively dry mud; The shrimp, *P. paucidens*, was harvested in the Lake of Ikeda (fresh water), Kagoshima [19]. *C. sapidus* a north east mediterranian crab (Celik *et al.,* 2004). *V. litterata*, an Indian freshwater crab [9]

5. DISCUSSION

Lipids are the principal storage forms of energy in many organisms including crabs and human. Different type of lipids, although present in relatively small quantities, play crucial roles as enzyme cofactors, electron carrier, light absorbing pigments, hydrophobic anchor for proteins, 'chaperons' to help membrane protein fold and emulsifying agents in digestive tract, hormones, and intracellular messengers. Lipids and their fatty acids are also used for locomotion, spawning, migration and also used as an energy source for reproduction and structural components of membrane which maintains the lipid homeostasis in the crabs.

Dietary lipids of man, which are at the focus of the investigation, are the structural and storage lipids of the animals and plants that form the food and the diet of man. It is a known fact that crab is one of the main sources of protein in the diet of the common Indian household. It is also rich in lipids and fatty acid especially the essential fatty acids that are required in minute amount but are not synthesized in the human body.

Now a day's people of India and other countries change their food habit from fish to different invertebrate like muscles and crustacean *viz*., crabs, shrimps, etc. Because the crabs are sweet, delicious and rich in fatty acids and protein, most importantly their market price is very cheap relative to beef, pork, fish etc. customer are so many in number. The main point is that crab being the most important food source of these vital nutrients for human, a long-lasting interest in crab lipids stem from their abundance and their uniqueness should be encouraged. The result presented in this article on lipid and fatty acid classes of crabs is discussed in comparison to other crustacean along with their basic biochemistry and importance in human nutrition.

The total lipid content from various crustaceans was observed (Table 1) and it is seen that the value ranges from 0.21-31.9% among these; freshwater crab *V. litterata* shows 1.03% TL value which consider it as lean fish category. Whereas, it was seen that *P. vigil*, *M. rosenbergii* (Male); *M. rosenbergii* (female), *P. mansonia* have TL content of 4.12-6.34%, 3.35- 5.35%, 16.8-31.9%, 5.85% respectively, reflecting that all of them have high lipid content. The importance of lipids in crustacean physiology is that it is one of the major organic sources other than protein. Lipids in body flesh are used as energy source for locomotion, stored and transported to gonads for reproduction and utilized during spawning migration and actual spawning. According to Ackman's data [80,81] *Varuna litterata* can be judged as low fat lean crab and hence recommended as perfect count as low fat protein to the patients suffering from gastrointestinal difficulties and overweight [9].

Fatty acids are aliphatic monocarboxylic acids derived from, or contained in esterified form in an animal or vegetable fat, oil, or wax. Natural fatty acids commonly have a chain of 4-28 carbons (usually unbranched and even numbered), which may be saturated or unsaturated. Fatty acids exist free in the body (that is, they are unesterified) and also are found as fatty esters in more

complex molecules, such as triacylglycerols. Low levels of free fatty acids occur in all tissues, but substantial amounts sometimes can be found on the plasma, particularly during fasting. Plasma free fatty acids (transported by serum albumin) are in route from their point of origin (triacylglycerols of adipose tissue or circulating lipoproteins) to their site of consumption (most tissue). Free fatty acids can be oxidized by many tissues particularly liver and muscle to provide energy. Fatty acids are also structural components of membrane lipids. Fatty acids are attached to certain intracellular proteins to enhance the ability of those proteins to associate with membranes. Fatty acids are also precursors of the hormone-like prostaglandins. Esterified fatty acids, in the form of triacylglycerols stored in adipose cells, serve as the major energy reserve of the body. It is seen that the all crustaceans in this observation have a no. of important fatty acids present in their edible part.

Palmitic acid (C16:0) is probably the commonest saturated fatty acid and is found in virtually all animal and plant fats and oil. It is seen that among all crustaceans this is the predominant SFA and maximally found in *V. litterata*. Other crustaceans are ranged from 14.8-15.8% of total lipid. Ackman *et al*. [82] remarked that palmitic acid is the prime fatty acid at all evolutionary as well as tropic levels. In fish, the defense mechanism against microbial infections performed particularly by palmitic acids through the pathogen-associated molecular pattern and T-cell signaling [83]. In the crustacean body palmitic acid may help to defense against protozoan infection. Thus it is found in every crustacean flesh as a remarkable amount.

Stearic acid (C18:0) is also relatively common and may on occasion be more abundant than palmitic acid, especially in complex lipid. Palmitic acid is also high in the edible Indian freshwater crab *V. litterata*. The freshwater shrimp, *P. paucidens*, have lower stearic acid among all crustaceans. The combination of total C16 and total C18 are also compared among the crabs. Combination of these two fatty acid groups are favored as substrates for mitochondrial β-oxidation and catabolised via the TCA cycle to generate metabolic energy [84] required in reproduction.

MUFA or monounsaturated fatty acids also occur naturally in chain lengths from about C14 to C24 but, although they are characterized by having a single unsaturated bond, the position of the ethylene bond within the carbon chain can vary even within a specific chain length, so that there are considerably more species of monounsaturated fatty acids than those of saturated fatty acids. In all animal desaturation of fatty acids takes place in the endoplasmic reticulum of cells of particular tissues via an aerobic process utilizing Co-A linked substrates and requiring NADPH and O2, catalyzed by multi- component systems comprising NADPH-cytochrome b5 reductase, cytochrome b5 and terminal desaturase enzymes [85]. This reaction is particular physiological importance in that the monounsaturated products formed (16:1ω7 and 18:1ω9) have markedly lower melting points (phase transition temperature) than their saturated precursors (16:0, 18:0). Hence, ∆9 fatty acid desaturase provides a means of regulating the viscosity of cell membranes by altering the phase transition

temperatures of the fatty acids in their constituent phosphoglycerides. In this observation, among all edible crustaceans highest MUFA is found in freshwater shrimp *P. paucidens* have highest amount and prawn, *P. japonicus* have lowest MUFA. The mud crab *S. serrata* has 25% of MUFA but interestingly freshwater crab *V. litterata* has 29% value of MUFA. According to Muhamad and Mohamed [86], MUFAs appeared to be the major fatty acid class in freshwater fishes.

Linoleic acid (18:2ω6) is the commonest and simplest fatty acid among dienes and found in most plant and animal tissues. It is an essential fatty acid in animal diet as it cannot be synthesized by the animal yet is required for growth, reproduction and healthy development [87]. *C. sapidus*, a north east Mediterranean crab have less linoleic acid among all but freshwater crab *V. litterata* have highest, followed

by the prawn, *P. japonicus* and mud crab, *S. dehaani*. Linolenic acid (18:3ω3) is a major component of plant lipids, particularly of the photosynthetic tissues, but it is a significant component of crab lipid. This is an extremely important as the primary source of other polyunsaturated fatty acids. It is seen that freshwater shrimp *P. paucidens* do not have linoleic acid where as mud crab *S. dehaani* and freshwater crab *V. litterata* shows high amount of linolenic acid (Fig. 3).

EPA (20:5ω3) and DHA (22:6ω3), in particular, are found in all marine animal tissues as major component. Fish take up the ω-3PUFA from their food, as essential nutritional components, which they cannot synthesize *de novo*. Along with simplified food chain, the animals can perform limited chain elongation and desaturation of the dietary ω-3PUFA (Fig. 1 shows the relation between dietary PUFA, Tissue PUFA and eicosanoid product). For this reason, the herbivores (e.g. abalone, oyster, mussels) and low order carnivore (e.g. crustaceans) tend to contain more EPA and less DHA than high order carnivores, which in turn contain less EPA than DHA (e.g. tuna, mackerel, shark, squid, octopus) [88,89]. Some crustaceans also show high amount of EPA and DHA, showing similarity with previous reports (Fig. 3). It is reported that, addition of 2g / Day of EPA to slandered antipsychotic therapy was superior to the addition of a 2g / day to DHA in decreasing residual symptoms [90]. The authors conducted the study on 30 individuals, with bi- polar disorder consuming 6.2g / day EPA and 3.4g / day DHA, and they found significantly longer period of remission. The data on crustaceans compared in this article have sufficient amount of EPA and DHA especially *Varuna litterata* which shows more or less considerable amount of these important PUFAs. Thus intake of only two servings/week to minimize the symptoms of schizophrenia and regular use of this food may give advance protection to schizophrenia.

The n-3/n-6 ratio is also addressed in this article. It has been suggested that n-3/n-6 ratio of 1.1 to 1.5 would contribute to a healthy human diet [91] and recommendation by WHO is that the n-3/n- 6 ratio in total human diet should be more than 1.5/day [92,93]. It is seen that among the crustaceans under discussion the freshwater crab *V. litterata*, maintains the recommended ratio, making it as an important food source for human for an advanced protection of cardio vascular disease (CVD).

6. CONCLUSION

It can be concluded that freshwater crab possess no less nutritional quality than other edible crustacean like marine crab, prawn, shrimp, etc. They are equally important for human consumption and have sufficient nutritional value and therapeutic value for cardiac heart disease patients. Crustaceans especially flesh of fresh water crabs, like *Varuna litterata,* has good quality of lipids and remarkably valuable low fat type fatty acid makes them lean shellfish. Being very cheap these can be used as marine counterpart. A few research works is performed on freshwater crabs in India, so more attention should be given on Indian freshwater crabs. Conservation strategy should be undertaken for locally important shellfish such as *V. litterata*. Taking freshwater crab or other crustacean in daily diet may results in better and advanced protection against cardiac heart disease and other lifestyle related disease. Exploration of the nutritional quality of freshwater crab will encourage farmers to promote freshwater crab culture in India. As well as the consumption of freshwater crab may help to prevent nutritional deficiencies in future and the topic is important for that.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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An Overview of Nutritional Composition and Potential Health Benefits of *Moringa sp.*

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ABSTRACT

Moringa, scientifically known as Moringa oleifera, is a multipurpose tree that has gained considerable attention due to its exceptional nutritional and medicinal properties. Native to the Indian subcontinent, Moringa is now cultivated in various tropical and subtropical regions worldwide. This article provides an overview of the nutritional composition and potential health benefits of Moringa, highlighting its role as a valuable resource for combating malnutrition and addressing various health conditions. Moringa leaves offer multiple health advantages, including antioxidant activity, anti-microbial activity, anti-cancerous activity, anti-inflammatory action, and many more. Hypertension, diabetes, heart failure, and several pathological situations are the cause of this oxidative stress. Natural antioxidants are always the first choice of consumers and are better than synthetic antioxidants resistance strain incidences of pathogens increase, resulting in higher death rates worldwide, and new and improved antimicrobial drugs must be developed. In this regard, medicinal plants are coming into the limelight, having a superior approach to health and being devoid of synthetic pharmaceutical side effects. The anticancer activity of Moringa leaves aqueous extracts was investigated against human hepatocellular carcinoma HepG2 cells. A significant reduction (44–52%) was seen in HepG2 cell growth when the leaf extracts were orally administered, making them potent anticancer agents. The information presented here is based on a review of relevant research articles available on Google Scholar.

Keywords: Antioxidant; anticancer; antimicrobial hypertension; anti-inflammatory action; medicinal plants; subtropical regions; Moringa oleifera; ayurvedic medicine.

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1. INTRODUCTION

Moringa, scientifically known as Moringa oleifera, is a tree native to the Indian subcontinent but is now cultivated in various tropical and subtropical regions worldwide [1-3]. It is widely cultivated for the diversified use of its young seed pods and green leaves as vegetables and for medicine [4-7]. It is considered as a very good supplement because of its high protein value. The species is highly regarded for its exceptional nutritional composition, making it a valuable dietary resource [8-13]. Moringa, a drought-resistant tree, has been traditionally used for centuries in Ayurvedic medicine. Recent scientific studies have further elucidated its therapeutic potential, revealing its rich content of vitamins, minerals, and bioactive compounds [14-20]. This section provides an introduction to Moringa and its historical significance.

2. HISTORY

The history of Moringa dates back thousands of years, and the plant has been recognized for its valuable properties in various cultures around the world. Native to the Indian subcontinent, Moringa oleifera, also known as the drumstick tree or horseradish tree, has a rich historical significance in Ayurvedic medicine and traditional healing practices. Moringa is a species of medicinal plant that has been used customarily to cure lesions and numerous diseases such as colds and diabetes. Sometimes making of paste with leaves or extractions of leaves was used. In addition, the type is also expended as a source of nutrients and extensively used for purging water.

- **Ancient Origins:**

Moringa's origins can be traced back to ancient India, which was highly regarded for its medicinal and nutritional properties. Historical texts and Ayurvedic scriptures mention the use of Moringa leaves, seeds, and roots in the treatment of various ailments, including digestive disorders, respiratory issues, and skin problems.

- **Spread across Ancient Civilizations:**

The fast-growing, drought-resistant moringa (Moringa oleifera) tree is native to South Asian nations such as India, Pakistan, Bangladesh, and Afghanistan and widely cultivated in tropical and subtropical countries.

As human populations expanded and trade routes developed, Moringa spread to other parts of Asia, Africa, and the Mediterranean. It found its way into the traditional healing practices of civilizations such as the Egyptians, Greeks, and Romans.

- **Historical Uses:**

Moringa Oleifera has its place in the Moringaceae family of plants that are native to Asia and Africa. The origins are not accurately obvious, but it's generally

assumed the Moringa tree was first determined and plowed for persistence in Northern India around 2000bc

In ancient Egypt, Moringa oil was used in perfumes and cosmetics due to its moisturizing and emollient properties. The Greeks and Romans used Moringa leaves as a nutritious food source and for medicinal purposes, recognizing its ability to boost energy and alleviate various health conditions.

- **Cultural Significance:**

Moringa is an important harvest in India, Ethiopia, the Philippines, and the Sudan, and is being grown in West, East, and South Africa, tropical Asia, Latin America, the Caribbean, Florida, and the Pacific Islands.

Moringa holds cultural significance in many regions. In Indian traditions, the tree is considered sacred and is often planted near temples and homes. Its leaves are used in religious rituals and festivals, symbolizing fertility, abundance, and well-being.

- **Colonial Era:**

During the colonial era, Moringa gained attention from European botanists and explorers. Its nutritional properties were highly valued as a potential food source for European settlers and soldiers. Moringa's hardiness and ability to grow in diverse climates made it a valuable resource during long voyages and in regions with limited agricultural options.

- **Modern Scientific Exploration:**

In the 20th century, scientific research on Moringa began to uncover its nutritional and medicinal potential. Scientists analyzed its chemical composition and studied its bioactive compounds, leading to a deeper understanding of its health benefits and practical applications. The Moringa genus encompasses 13 species disseminated via southwest Asia, southwest Africa, northeast Africa, and Madagascar.

- **Contemporary Cultivation:**

Today, Moringa is cultivated in various tropical and subtropical regions worldwide, including parts of Asia, Africa, and the Americas. Its leaves, pods, and seeds are utilized for food, medicine, and commercial purposes. Moringa plantations and initiatives have been established in developing countries to combat malnutrition and address food security challenges.

- **Global Recognition:**

Moringa has gained recognition and popularity in recent years due to its nutrient density, adaptability to diverse environments, and potential for sustainable

agriculture. It has been featured in numerous health and wellness publications, promoting its role as a "super food" and contributing to its global demand.

Moringa leaves have shown an emetic effect, making Moringa an imaginable possibility for irregularity. In one study, Moringa abridged stomach acidity by around 85%, suggesting it could avert peptic ulcers. Moringa's antibiotic and antibacterial properties may help prevent the growth of pathogens that can cause infections.

The rich history of Moringa showcases its enduring significance in various cultures and highlights the ongoing exploration of its nutritional and medicinal potential. As scientific research continues to unveil its remarkable properties, Moringa's legacy as a versatile and valuable plant persists in modern times.

3. NUTRITIONAL COMPOSITION OF MORINGA

Moringa leaves, pods, seeds, and roots are rich sources of essential nutrients, including proteins, vitamins (A, C, E, and B-complex), minerals (calcium, iron, and potassium), and dietary fiber. It is particularly known for its exceptional protein content and diverse range of antioxidants. Moringa oleifera seeds are widely used in water and effluent treatment, for their coagulation, flocculation, and sedimentation properties, as their ability to improve water quality, by reducing organic matter and microbial load, with special applicability in intensive animal production systems, such as aquaculture. In addition, due to its high nutritional value and several medicinal properties, this tree may act as a nutritional and medical alternative for socially neglected populations.

- **Leaves:**

Moringa leaves are the most commonly consumed part of the plant and are rich in essential nutrients. They are a good source of protein, containing all nine essential amino acids, making them a complete protein source for vegetarians and vegans. The leaves are also packed with vitamins, including vitamin A (as beta-carotene), vitamin C, vitamin E, and a range of B vitamins (such as thiamin, riboflavin, niacin, and folate). Additionally, Moringa leaves are abundant in minerals, including calcium, iron, magnesium, potassium, and zinc. EAA – essential amino acids – threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine, arginine, and tryptophan.

- **Pods:**

The immature green pods of Moringa, often referred to as drumsticks, are consumed as a vegetable. They are low in calories but rich in nutrients. Moringa pods are a good source of vitamin C, vitamin A, and vitamin B-complex, particularly niacin and vitamin B6. They also provide dietary fiber, which aids digestion and promotes a healthy gut.

- **Seeds:**

Moringa seeds are contained within the pods and have a unique nutritional profile. They are rich in healthy fats, including monounsaturated and polyunsaturated fats. These fats contribute to the maintenance of healthy cholesterol levels. Moringa seeds are also a good source of protein, containing significant amounts of essential amino acids. Moreover, they contain beneficial compounds like antioxidants and phenolic compounds, which provide various health benefits.

- **Roots:**

While less commonly consumed than the leaves and pods, Moringa roots are also utilized for their nutritional properties. The roots are a good source of vitamins, particularly vitamin C, and minerals like potassium and iron. They are also known to contain alkaloids, flavonoids, and other bioactive compounds with potential health benefits.

Nutritional Density:

Moringa's exceptional nutritional composition has led to its classification as a "super food." It is considered one of the most nutrient-dense plants on the planet, containing high levels of vitamins, minerals, and antioxidants. The combination of its protein content, essential amino acids, and diverse range of nutrients makes Moringa a valuable addition to a balanced diet.

In summary, Moringa is a nutritionally rich tree that offers a wide array of essential nutrients. Its leaves, pods, seeds, and roots provide a diverse range of vitamins, minerals, proteins, and antioxidants, making it an excellent dietary resource. Incorporating Moringa into one's diet can contribute to overall nutrition and promote well-being.

4. ROLE OF MORINGA IN MALNUTRITION

The role of Moringa in addressing malnutrition has gained significant attention, particularly in developing countries where malnutrition is a pressing issue. Moringa's nutritional density and availability make it a valuable resource in combating malnutrition. Here are some key aspects of Moringa's role in addressing malnutrition:

- **Nutrient-Rich Composition:**

Moringa is packed with essential nutrients, including proteins, vitamins, minerals, and antioxidants. Its leaves, pods, and seeds contain a wide range of vitamins, such as vitamin A, vitamin C, and various B vitamins. It also provides important minerals like calcium, iron, potassium, and zinc. These nutrients are crucial for the overall growth, development, and maintenance of the human body.

- **Protein Source:**

Moringa leaves are known to contain high-quality proteins with all nine essential amino acids. Proteins are essential for building and repairing tissues, supporting immune function, and producing enzymes and hormones. Incorporating Moringa into diets can help address protein deficiencies, particularly in regions where animal protein sources are limited.

- **Addressing Micronutrient Deficiencies:**

Moringa's nutrient profile makes it a valuable tool in combating micronutrient deficiencies, such as vitamin A, iron, and zinc deficiencies. These deficiencies can lead to impaired growth, compromised immune function, and increased susceptibility to diseases. Regular consumption of Moringa can help supplement these essential micronutrients, promoting optimal health and reducing the risk of associated deficiencies.

- **Accessible and Culturally Acceptable:**

Moringa is often available and accessible in areas where malnutrition is prevalent. It is relatively easy to cultivate, even in arid and semi-arid regions, and its parts (leaves, pods, and seeds) can be easily incorporated into local diets. Moringa's cultural acceptance and familiarity in certain regions make it more likely to be embraced as a food source.

- **Sustainable Agriculture:**

Moringa is known for its resilience and ability to grow in diverse environmental conditions. It requires minimal water and can withstand dry spells, making it suitable for regions with water scarcity. The cultivation of Moringa trees can contribute to sustainable agriculture practices, providing a renewable source of nutrition to combat malnutrition in communities.

- **Community Empowerment:**

Moringa cultivation and utilization can also contribute to community empowerment. Small-scale farming and processing of Moringa products, such as leaf powder or seed oil, can provide income-generating opportunities for local communities. This not only improves nutrition but also enhances livelihoods and economic stability.

While Moringa alone cannot solve the complex issue of malnutrition, it can play a valuable role in supplementing diets with essential nutrients and addressing specific nutritional deficiencies. Its availability, nutritional density, and adaptability make it a promising resource in the fight against malnutrition, particularly in resource-constrained settings. However, it is essential to consider Moringa's integration into broader nutrition programs and ensure that its consumption is part of a diversified and balanced diet for long-term impact.

5. ANTI-INFLAMMATORY AND IMMUNOMODULATORY EFFECTS OF MORINGA

Moringa exhibits notable anti-inflammatory and Immunomodulatory effects, which contribute to its potential therapeutic applications.

Anti-Inflammatory Effects:

Moringa possesses several bioactive compounds, including flavonoids, phenolic acids, and isothiocyanates, which contribute to its anti-inflammatory properties. These compounds help to reduce inflammation in the body through various mechanisms:

- **Inhibition of Inflammatory Mediators:**

Moringa is a genus of shrubs and trees with multi-persistence uses: its leaves, roots, and small pods are used as a vegetable. All parts of the Moringa tree – bark, pods, leaves, nuts, seeds, tubers, roots, and flowers – are edible. The leaves are consumed as fresh or dried and ground into powder.

Moringa compounds, such as quercetin and kaempferol, have been shown to inhibit the production and release of pro-inflammatory mediators, such as cytokines (e.g., TNF-alpha and interleukins) and prostaglandins. By reducing the production of these inflammatory molecules, Moringa helps to modulate the inflammatory response.

- **Suppression of Inflammatory Pathways:**

Moringa can inhibit the activation of nuclear factor-kappa B (NF-κB), a key transcription factor involved in the regulation of inflammation. NF-κB activation triggers the expression of inflammatory genes. By inhibiting NF-κB, Moringa helps to down regulate the inflammatory signaling pathways.

- **Antioxidant Activity:**

Oxidative stress plays a role in promoting inflammation. Moringa is rich in antioxidants, including vitamin C, vitamin E, and various phenolic compounds, which help to neutralize harmful free radicals and reduce oxidative stress. By reducing oxidative stress, Moringa indirectly mitigates inflammation.

Immunomodulatory Effects:

Moringa also exhibits Immunomodulatory effects, meaning it can regulate and modulate the immune response. These effects are mediated by several bioactive compounds present in Moringa, such as phenolic acids, flavonoids, and alkaloids. Here's how Moringa's Immunomodulatory properties work:

- **Enhancing Immune Function:**

The leaves of Moringa Oleifera are mostly used for medicinal purposes as well as for human nutrition, since they are rich in antioxidants and other nutrients, which are generally lacking in people living in emergent countries [21]. Moringa leaves have been used for the management of various diseases from malaria and typhoid fever to hypertension and diabetes [22].

Leaves of the Moringa tree have been observed to encompass flavonoids containing myricetin, quercetin, kaempferol, isorhamnetin, or rutin, as well as phenolic acids. Fresh leaves are a good source of carotenoids such as lutein, β-carotene, and zeaxanthin.

Moringa extracts have been found to enhance the activity and proliferation of immune cells, including T cells, B cells, and natural killer (NK) cells. These immune cells play crucial roles in combating infections, defending against pathogens, and maintaining immune system balance.

- **Anti-Allergic Effects:**

Moringa has shown potential in reducing allergic reactions. It can suppress the release of histamine, a key mediator involved in allergic responses, and inhibit the activation of mast cells, which play a central role in allergic inflammation.

- **Balancing the Immune Response:**

Flavonoids (apigenin, quercetin, luteolin, myricetin, kaempferol), lignans (secoisolariciresinol, isolariciresinol, medioresinol, epipinoresinol glycosides), and phenolcarboxylic acids and their derivatives (coumaroylquinic, caffeoylquinic, feruloylquinic acids) are the main phenolic compounds found in Moringa leaves.
Moringa compounds can modulate the immune response by promoting a balanced immune reaction. They have been shown to regulate the production of pro-inflammatory and anti-inflammatory cytokines, helping to maintain immune homeostasis.

- **Antimicrobial Defense:**

Ethanolic extract of Moringa leaf demonstrated antibacterial effects counter to food-borne pathogens.

Moringa's Immunomodulatory effects also include antimicrobial activity. By enhancing immune function, Moringa supports the body's natural defense mechanisms against infections caused by bacteria, viruses, and fungi.

The anti-inflammatory and Immunomodulatory effects of Moringa contribute to its potential therapeutic applications in various inflammatory and immune-related conditions. However, it's important to note that further research is needed to fully understand the underlying mechanisms and optimize the use of Moringa in clinical settings.

6. ANTIDIABETIC PROPERTIES OF MORINGA

Moringa possesses anti diabetic properties, which have been attributed to various bioactive compounds present in the plant. Here's an explanation of the anti-diabetic effects of Moringa:

- **Regulation of Blood Glucose Levels:**

Moringa leaves have Quercetin which is an antioxidant that helps to lower blood pressure and an alternative antioxidant is Chlorogenic acid which alleviates blood sugar levels. The Chlorogenic acid found in moringa may help the body progress sugar better and affect insulin too.

Moringa has been found to have hypoglycemic properties, meaning it helps to lower blood glucose levels. This effect is particularly beneficial for individuals with diabetes. The anti-diabetic properties of Moringa are attributed to several mechanisms:

- **Increased Insulin Secretion:**

Studies have shown that Moringa extracts can stimulate insulin secretion from pancreatic beta cells. Insulin is a hormone responsible for regulating blood sugar levels by facilitating the uptake of glucose into cells. By promoting insulin secretion, Moringa helps to enhance glucose utilization and reduce blood glucose levels.

- **Enhanced Insulin Sensitivity:**

Moringa has been shown to improve insulin sensitivity, which is the ability of cells to respond to insulin effectively. Improved insulin sensitivity allows for better glucose uptake by cells, thereby reducing blood glucose levels. This effect is beneficial for individuals with insulin resistance, a condition often associated with type 2 diabetes.

- **Inhibition of Glucose Absorption:**

Moringa contains compounds, such as chlorogenic acid and quercetin, which have been found to inhibit the enzymes responsible for breaking down carbohydrates into glucose. By inhibiting these enzymes, Moringa can reduce the absorption of glucose from the digestive system, leading to lower blood sugar levels.

- **Antioxidant Activity:**

Oxidative stress plays a significant role in the development and progression of diabetes. Moringa is rich in antioxidants, such as vitamin C, vitamin E, and various phenolic compounds, which help to neutralize harmful free radicals and reduce oxidative stress. By reducing oxidative stress, Moringa protects

pancreatic beta cells and improves their function, contributing to better glucose regulation.

- **Anti-Inflammatory Effects:**

Chronic inflammation is closely associated with insulin resistance and the development of type 2 diabetes. Moringa's anti-inflammatory properties, as discussed earlier, help to reduce systemic inflammation. By mitigating inflammation, Moringa supports insulin sensitivity and improves overall glucose control.

- **Lipid Profile Regulation:**

Diabetes is often accompanied by abnormalities in lipid metabolism, leading to dyslipidemia (elevated levels of cholesterol and triglycerides). Moringa has been shown to help regulate lipid profiles by reducing total cholesterol, LDL cholesterol, and triglyceride levels while increasing HDL cholesterol levels. These effects contribute to better cardiovascular health and help manage the complications associated with diabetes.

It's important to note that while Moringa shows promise as an antidiabetic agent, it should not replace conventional diabetes management strategies. Moringa can be incorporated into a balanced diet and lifestyle changes recommended for diabetes management, under the guidance of a healthcare professional.

7. ANTIMICROBIAL ACTIVITY OF MORINGA

Moringa exhibits notable antimicrobial activity against a wide range of microorganisms, including bacteria, viruses, and fungi. The antimicrobial properties of Moringa can be attributed to various bioactive compounds present in the plant. Here's an explanation of the antimicrobial activity of Moringa:

Antibacterial Effects:

Moringa extracts have demonstrated significant antibacterial activity against both gram-positive and gram-negative bacteria. The antibacterial effects are attributed to multiple mechanisms:

- **Disruption of Cell Membrane:**

Certain bioactive compounds in Moringa, such as isothiocyanates, have been shown to disrupt the cell membrane integrity of bacteria. This disrupts their structural integrity and function, leading to bacterial cell death.

- **Inhibition of Bacterial Growth:**

Moringa extracts have been found to inhibit the growth of various bacteria by interfering with their metabolic pathways. These include enzymes involved in cell wall synthesis, DNA replication, and protein synthesis, among others.

- **Antioxidant Activity:**

Moringa's antioxidant properties, attributed to compounds like phenolic acids and flavonoids, contribute to its antibacterial effects. Antioxidants help neutralize harmful free radicals and oxidative stress, which can weaken bacterial cells and inhibit their growth.

Antiviral Effects:

Moringa exhibits antiviral activity against several types of viruses. The antiviral effects of Moringa can be attributed to the following mechanisms:

- **Viral Replication Inhibition:**

Moringa extracts have been found to inhibit the replication of certain viruses by interfering with their viral enzymes and inhibiting the expression of viral proteins. This prevents viral replication and the spread of infection.

- **Immunomodulatory Effects:**

Moringa's immunomodulatory properties, as mentioned earlier, can contribute to its antiviral effects. By enhancing immune function, Moringa aids in the body's defense against viral infections, promoting the clearance of viruses.

Antifungal Effects:

Moringa extracts have shown antifungal activity against various fungal species, including Candida and Aspergillus. The antifungal effects are attributed to:

- **Disruption of Fungal Cell Membrane:**

Similar to its antibacterial effects, certain bioactive compounds in Moringa can disrupt the cell membrane of fungal cells, leading to their death.

- **Inhibition of Fungal Growth:**

Moringa extracts have been found to inhibit the growth of fungi by interfering with their metabolic processes, such as ergosterol synthesis, which is crucial for fungal cell membrane integrity.

- **Antioxidant and Immunomodulatory Effects:**

Moringa's antioxidant and Immunomodulatory properties also contribute to its antifungal effects. By reducing oxidative stress and enhancing immune function, Moringa helps the body combat fungal infections.

8. MEDICINAL PROPERTIES OF MORINGA

The guidelines set by the Indian Council of Medical Research (ICMR) and the World Health Organization (WHO) recommended that herbal medications, herbs, spices, and nutrients can help accomplish Covid-19 by enhancing immunity in patients. The role of herbs as Immunomodulatory is like a two-edged knife. On the one hand, it can function as an immunosuppressant desirable by a positive covid-19. But on the other hand, the immunostimulant activity of herbal medicines is mandatory to upsurge immunity which helps prevent Covid-19. Therefore therapy with herbal medicine must be used sensibly in terms of the types of plant that were used in the formulation, secondary metabolites, and the amount [23].

Herbal medicines for therapeutic resolutions have been explicitly used since the dawn of human development to preserve health and to cure diseases. Crude herbals and pharmaceutical substances of herbal origin are widely utilized in various types of pharmaceutical formulations.

Moringa possesses countless medicinal properties, which have been acclaimed, and experiments for their potential therapeutic applications. Here's an explanation of the medicinal properties of Moringa:

- **Cholesterol-lowering effects:**

Moringa has shown promise in reducing cholesterol levels, particularly LDL (low-density lipoprotein) cholesterol, also known as "bad" cholesterol. This effect is attributed to compounds like beta-sitosterol and quercetin, which help inhibit cholesterol absorption and promote its elimination from the body. By improving lipid profiles, Moringa may contribute to cardiovascular health.

- **Hepatoprotective Properties:**

Research suggests that Moringa has hepatoprotective effects, meaning it can protect the liver from damage. Its antioxidant and anti-inflammatory properties play a role in reducing liver inflammation and oxidative stress. Moringa may also support liver function by promoting the detoxification process and enhancing the production of enzymes involved in liver metabolism.

- **Wound Healing and Skin Benefits:**

Moringa contains bioactive compounds that promote wound healing, including anti-inflammatory and antimicrobial properties. It may help accelerate the healing process, reduce inflammation, and prevent infection. Additionally, Moringa oil is rich in nutrients and antioxidants, making it a potential ingredient for skincare products to nourish and protect the skin.

- **Cardiovascular Health:**

Certain components of Moringa, such as flavonoids and polyphenols, have been associated with potential cardiovascular benefits. They may help lower blood

pressure, reduce cholesterol levels, prevent plaque formation in blood vessels, and improve overall heart health. These properties suggest that Moringa could have a protective effect against cardiovascular diseases.

- **Antioxidant Activity:**

Moringa is rich in antioxidants, including vitamins C and E, flavonoids, and phenolic compounds. These antioxidants help neutralize harmful free radicals, which can cause oxidative stress and damage cells. The antioxidant activity of Moringa contributes to its potential benefits in reducing inflammation, preventing chronic diseases, and supporting overall cellular health.

- **Anti-asthmatic Activity:**

Moringa oleifera seed kernels used in bronchial asthma have explained simultaneous development in respiratory functions and an outstanding decrease in the severity of symptoms of asthma, therefore the study reported the efficacy and safety of the seeds. Methanolic extract of Moringa oleifera leaves exerted bronchodilator effects such as blocking the release of inflammatory mediators into the local lung tissues and inhibiting inflammatory mediators such as histamine at a dose of 250 mg/kg and 500 mg/kg. The study confirmed that Moringa oleifera leaves had antiasthmatic activity. The most phytochemical constituents consisted of the leaves compared to seeds of Moringa oleifera; therefore, higher phytochemical compounds have encouraged the study to evaluate the anti-asthmatic effects using dexamethasone as the standard drug. The result obtained suggested that Moringa oleifera leaf possessed a great effect against mast cell degranulation, anti-inflammation, and bronchospasm. Fidrianny et al. indicated that Moringa oleifera Lam seeds in animals investigational as an antiasthma were used at a dose of 250 mg/kg BW and 500 mg/kg BW. The use of these herbs had shown a progress in respiratory function and a reduction in the relentlessness of asthma signs. The safety of the formulation was rated as good. Methanol extract of Moringa oleifera Lam seeds had a bronchodilator effect by blocking the release of inflammatory mediators into local lung tissue and preventing inflammatory mediators such as histamine [24].

- **Anti-inflammatory Effects:**

Moringa exhibits significant anti-inflammatory properties. It contains compounds like flavonoids, isothiocyanates, and phenolic acids, which can help reduce inflammation in the body. Chronic inflammation is linked to various health conditions, including cardiovascular diseases, diabetes, and certain cancers. Though all parts of the moringa plant have been used traditionally, moringa seeds have specifically been reported to possess anti-inflammatory, antioxidant, hypotensive, antibacterial and chemopreventive properties. The anti-inflammatory effects of Moringa make it beneficial for managing and preventing such conditions.

- **Antimicrobial Activity:**

Moringa possesses antimicrobial properties against bacteria, viruses, and fungi. The bioactive compounds in Moringa, such as isothiocyanates and phenolic acids, contribute to its antimicrobial activity. These properties make Moringa potentially useful in combatting bacterial and fungal infections and supporting immune function against viral pathogens.

- **Anticancer Potential:**

Several studies have suggested that Moringa exhibits anticancer properties. Its bioactive compounds, such as quercetin, kaempferol, and glucosinolates, have shown potential in inhibiting the growth of cancer cells and inducing apoptosis (programmed cell death) in various types of cancer. However, further research is needed to better understand the mechanisms and potential applications of Moringa in cancer treatment.

- **Nutritional Supplementation:**

In addition to its medicinal properties, Moringa is highly nutritious and contains essential vitamins, minerals, proteins, and antioxidants. It can serve as a valuable dietary supplement to address nutrient deficiencies and support overall health and well-being.

9. SAFETY AND POTENTIAL SIDE EFFECTS OF MORINGA

Moringa is generally considered safe for consumption when used in appropriate amounts. However, like any dietary supplement or natural product, it is essential to be aware of potential side effects and consider individual circumstances. Here's an explanation of the safety and potential side effects of Moringa:

- **Allergic Reactions:**

Some individuals may be allergic to Moringa. Allergic reactions can range from mild symptoms, such as skin rash and itching, to more severe reactions like difficulty breathing or anaphylaxis. If you have a known allergy to plants in the Moringaceae family, it is advisable to avoid Moringa.

- **Digestive Issues:**

Consuming large amounts of Moringa or taking concentrated extracts may cause digestive issues in some individuals. These can include diarrhea, stomach cramps, and nausea. It is recommended to start with small amounts of Moringa and gradually increase the dosage to assess individual tolerance.

- **Interference with Medications:**

Moringa may interact with certain medications. It contains compounds that can potentially affect the metabolism of drugs processed by the liver's cytochrome

P450 enzymes. If you are taking any medications, especially those with narrow therapeutic indices, it is advisable to consult a healthcare professional before using Moringa as a dietary supplement.

- **Blood Sugar Regulation:**

Moringa has hypoglycemic properties and may lower blood sugar levels. Individuals with diabetes or hypoglycemia should monitor their blood sugar levels closely when incorporating Moringa into their diet to prevent hypoglycemia. Adjustments to diabetes medication may be necessary under the guidance of a healthcare professional.

Thus, the anti-diabetic properties of the Moringa plant have attracted high attention from researchers. In the last twenty years, a massive number of new chemical structures and their pharmacological activities have been reported specifically the anti-diabetic properties.

- **Pregnancy and Breastfeeding:**

There is limited scientific evidence regarding the safety of Moringa during pregnancy and breastfeeding. Pregnant and breastfeeding women should consult their healthcare provider before using Moringa as a dietary supplement.

- **Contaminants:**

As with any plant-based product, there is a possibility of contamination with microbes, heavy metals, or other pollutants. It is important to choose Moringa products from reputable sources that adhere to quality control standards to minimize the risk of contamination.

It's worth noting that the side effects and safety considerations mentioned above are based on limited scientific studies. Moringa has been consumed as a food source for centuries in many cultures without reported significant adverse effects. However, individual responses may vary, and it's always prudent to exercise caution and seek professional advice when considering any dietary supplement.

The utilization of traditional medicinal herbs can support efforts to increase living healthily and independently.

If you have any specific health concerns or are considering using Moringa for a specific medical condition, it is recommended to consult a healthcare professional for personalized guidance and to ensure its safe use.

10. CONCLUSION

The plants have always been dynamic for mankind regardless of the era and area all over the globe since the establishment of life. These were, are and will remain ever helpful from nutritional, social, cultural, religious, environmental and

human's health etc. The medicinal plant have utmost prospective for benefitting people, especially those living in countries {like Pakistan, India} suffering from poverty, poor health, malnutrition, redundancy and isolation in international employment. Three technologies namely "Biotechnology", "Herbal technology" and "Information technology" are going to be the most powerful components that are crucial for prosperity and health for the people of nations. All technologies for the production of value added plant products can be called as herbal technology. In addition, some studies have found that the compounds in Moringa Oleifera may also defend against Alzheimer's disease and Parkinson's disease. Moringa has been used in the customary medicine passed down for epochs in many cultures everywhere in the word, for skin infections, anemia, anxiety, asthma, blackheads, blood impurities, bronchitis, mucus, chest congestion, cholera.Moringa oleifera is a noticeable source of nutrients and antioxidants. Like other vegetables such as spinach and fenugreek, Moringa leaves are not as popular all over the world, but currently, it is used as alternates in soups, lentils, and other preparations in Southeast Asia. Still there is a knowledge breach in potential uses of Moringa as a food supplement and food fortification. Moringa has massive potential uses but is very less reconnoitred. It can be consumed to make foods that could be a step towards decrease malnutrition. The published literature gives the total scenario of the chemical constituents, nutritional content, and potential uses of the plant. The identification, isolation, and calibration of plant extracts may be considered for comprehensive studies which can be beneficial for the further expansion of the auspicious food products with health reimbursements and nutrients to cure different life style-related illnesses as well as undernourishment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Research Area: Her research area mainly includes the following:

- Relationship between Smoking and Diet
- The Effects of Menopause and Diet
- Cultural Food Tradition in Kashmir
- Difference between Food Allergy and Sensitivity
- Study on the Effects of Fast Food on Humans
- Ways to Stop Worrying about Food and Weight Loss
- Ketogenic Diet, its Benefits and Overall Effects on Adults
- Archives of Clinical Case Reports Regarding Vitamin D Deficiency and Risk of Neuropsychiatric Disorders
- Iron Supplementation during Pregnancy -The Most Needed but the Most Neglected Element of Antenatal Care
- Role of Nutrition Therapy during COVID-19
- Childhood Obesity, its Factors, Complications and Impact on Overall Health in Children

Number of Published papers: She has published 19 articles in reputed journals.

Special Award: She has received the following awards:

- Honoured with a Laptop for excellent performance on community health and nutrition
- Community-Based Programmes Special Award
- Urdu Poetry Book Publication Award

Any other remarkable point(s): She has dedicated her life to learning and educating others regarding Health Sciences. She is an enthusiastic individual who aspires to offer counselling and courses to people in order to acquaint them with a healthy lifestyle and create a better environment for themselves by constructing a peaceful relationship with food. Her MPhil thesis was based on the Effects of Media on Food Choices of university students. She hopes that her social work revolving around heart patients, diabetic children under the age of five and the geriatric population influences her community and work positively so that more people are aware of a balanced diet leading to a healthy lifestyle. She is also mindful of carrying out internships with her students in famous food districts and helping them with dietary advice.

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- Disease Management Attitudes & Nutritional Status of Diabetes Mellitus Patients in AJ&K.
- Childhood obesity.
- Ketogenic Diets.
- Menopause and Diet.
- Cultural Food Traditions in Kashmir.

Number of Published papers: She published 14 research papers and 1 book chapter in reputed journals.

Special Award: She got an appreciation from her University administration.

Any other remarkable point(s): She is a motivated and workaholic person who takes a deep interest in the latest trends in Nutritional Sciences and its associated fields, is an avid reader of new research and takes initiative to contribute to the desired field. She is determined to produce desirable results in the relevant field. Objectively assessing, the needs of society and achieving desired goals, every possible effort is made to contribute to the betterment of society.

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CRISPR: Gene Editing's Future

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ABSTRACT

Genome editing has revolutionized molecular biology, with the Clustered Regularly Interspaced Short Palindromic Repeats and their associated protein Cas-9 (CRISPR-Cas9) system emerging as a pivotal tool. Originating from bacterial adaptive immune systems, CRISPR has evolved into a versatile genome editing mechanism, surpassing previous techniques in efficiency and accessibility. The CRISPR-Cas-9 system is a highly effective and precise genome editing tool utilized across various fields. The history of CRISPR spans decades, from its initial discovery in bacteria to its transformative applications in mammalian genome editing. The CRISPR-Cas9 system comprises two essential components: the Cas9 protein and guide RNA (gRNA). Together, they enable precise DNA cleavage and modification. CRISPR finds applications in medicine, biotechnology, and beyond, promising personalized treatments for genetic diseases and advancements in drug development and crop engineering. Recent advancements include the development of novel Cas proteins and improved editing techniques, driving the field forward. Efforts to enhance precision and reliability in gene editing continue to emphasize the ongoing commitment to unlock the full potential of CRISPR technology. In conclusion, CRISPR-Cas technology represents a transformative tool in biology, with ongoing efforts focused on refining its capabilities for precise and reliable gene manipulation. This paper provides an overview of the history, components, mechanisms, classifications, applications, advancements, and challenges of CRISPR technology.

Keywords: CRISPR; Cas9; gene editing; advancement.

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1. INTRODUCTION

Genome editing stands as a revolutionary field in molecular biology, with the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR-Cas9) system taking center stage and garnering significant attention [1]. CRISPR-Cas9 originated from a naturally occurring genome editing mechanism employed by bacteria for immune defense against viruses. When facing viral infections, bacteria capture fragments of the viruses' DNA, integrating them into their own DNA in a specific pattern to form CRISPR arrays. These arrays enable bacteria to "memorize" the viruses for future encounters. In the event of a repeat viral attack, the bacteria generate RNA segments from the CRISPR arrays, which recognize and bind to specific regions of the viruses' DNA. Subsequently, the bacteria utilize the Cas9 enzyme or a similar enzyme to cleave the DNA, effectively disabling the virus. Scientists have repurposed this natural immune defense system for DNA editing. They design a small RNA fragment containing a concise "guide" sequence that binds to a precise target sequence within a cell's DNA, akin to the RNA segments produced by bacteria from the CRISPR array. This guide RNA also associates with the Cas9 enzyme. Upon introduction into cells, the guide RNA identifies the designated DNA sequence, and the Cas9 enzyme performs a cut at the targeted location, mimicking the bacterial process. While Cas9 is the most commonly used enzyme, other enzymes like Cpf1 can also be employed. Following the DNA cut, researchers leverage the cell's inherent DNA repair machinery to either insert or remove genetic material or modify the DNA by substituting an existing segment with a tailored DNA sequence. The CRISPR-Cas9 system, known for its exceptional precision and versatility, has introduced a transformative era in genome editing, surpassing earlier techniques like zinc finger nucleases and transcription activator-like effector nucleases (TALENs) in terms of cost-effectiveness and user-friendliness, marking substantial progress in this domain. Its uses are varied, encompassing fields such as medical research, human gene therapy, plant science, and crop enhancement [2]. Fundamentally, the CRISPR-Cas9 system comprises two vital elements: the Cas9 protein and the guide RNA (gRNA). Originally adapted from bacterial adaptive immune systems, this molecular mechanism has been re-engineered for genome editing across different organisms, including humans, owing to its straightforwardness and versatility [3].

History of CRISPR:

1979	Gene Replacement in yeast.
1987	Escherichia coli was the first to discover CRISPR-grouped repeats. CRISPR-associated clustered repeats were identified. Ishino et al. conducted the first investigation on CRISPR clustered repeats (CRISPR) in 1987.
1993	The identification of CRISPR clustered repeats in M. tuberculosis.
2002	Identification of Cas genes, Coined the CRISPR acronym.
2005	Cas genes found in CRISPRs include virus sequences.

2008	CRISPR system types III-A target DNA and discovered the function of crRNA. The first experimental demonstration that CRISPR confers adaptive immunity (Barrangou et al., 2007) indicates that CRISPR loci may confer adaptive immunity on their hosts.
2010	Cas9 is led to the place where the DNA will be cut by protospacer sequences. Gameau et al. (2010) found that Cas9 cuts target DNA and that Type II CRISPR Cas also cuts target DNA
2011	Cas9 was discovered to be an RNA-guided endonuclease in living organisms, and its capacity to target DNA was established.
2012	Cas9 was demonstrated to be an RNA-guided endonuclease in living organisms, and its capacity to target DNA was described. Cas9-driven DNA targeting: a lab investigation. CRISPRCas9 is an RNA-directed DNA endonuclease that has been examined in a laboratory dish.
2014	Mammalian cells' genomes are being altered. Mammalian cells' genomes are being altered. The discovery of CRISPRa, CRISPRi, and dCas9.Cas9 RNA enables site-specific genome editing in human cells and other living organisms. The discovery of dCas9 CRISPRi and CRISPRa. Cas9RNA enables site-specific genome editing in human cells and other living organisms.
2015	Therapeutic proof of concept based on the discovery ofCas13a (C2c2)
2016	The Base Editor (BE) was created following the discovery of Cas13a (C2c2).
2018	1st CRISPR clinical trial for cancer immunotherapy.
2019	The first in vivo CRISPR clinical study was conducted for the treatment of monogenic diseases. The creation of nCATS using CRISPR/Cas9.
2020	Emmanuelle Charpentier and Jennifer A. Doudna got the Nobel prize for CRISPR Cas9 genome editing for their 2012 work on Crispr-Cas9, a method to edit DNA.

2. COMPONENTS

The CRISPR/Cas-9 system primarily comprises two essential elements: Guide RNA (gRNA) and CRISPR-associated (Cas-9) proteins. The initial Cas protein utilized in genome editing, Cas-9, is derived from Streptococcus pyogenes (SpCas-9). It serves as a substantial multi-domain DNA endonuclease, consisting of 1368 amino acids and acting as a genetic scissor, responsible for cleaving the target DNA to generate a double-stranded break. Cas-9 is divided into two key regions—the recognition (REC) lobe and the nuclease (NUC) lobe. The REC lobe, containing REC1 and REC2 domains, binds to the guide RNA, while the NUC lobe comprises RuvC, HNH, and Protospacer Adjacent Motif (PAM) interacting domains. The RuvC and HNH domains play a role in cutting each single-stranded DNA, and the PAM interacting domain confers PAM specificity, initiating binding to the target DNA. Guide RNA consists of two segments—CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). The crRNA, typically 18–20 base pairs in length, designates the

target DNA by forming pairs with the target sequence. On the other hand, tracrRNA is an extended sequence of loops that acts as a binding scaffold for the Cas-9 nuclease. In prokaryotes, guide RNA is utilized to target viral DNA. However, in the context of the gene-editing tool, it can be artificially designed by combining crRNA and tracrRNA to create a single guide RNA (sgRNA), allowing the targeting of virtually any gene sequence intended for editing [4].

3. HOW CRISPR WORKS

The Cas9 protein acts like molecular scissors, precisely cutting DNA at specific places. It has two parts that cut the DNA and another part that recognizes the gRNA. When Cas9 binds to both the gRNA and the target DNA, it changes its structure, creating a break in the DNA at the chosen spot. The gRNA is like a guide that directs Cas9 to the right place in the genome for editing. This RNA molecule has a scaffold part and a customizable guide sequence. The guide sequence is carefully designed to match the target DNA, helping Cas9 bind and cut the DNA. The step-by-step workings of the CRISPR-Cas9 system unfold systematically, encompassing tasks from pinpointing the target to precise modifications of genes. Examining cellular repair mechanisms, one can witness the swift corrective action of the Non-Homologous End Joining (NHEJ) pathway, swiftly mending DNA breaks. This pathway serves as a pathway for genetic progress, leading to transformative applications [5,6].

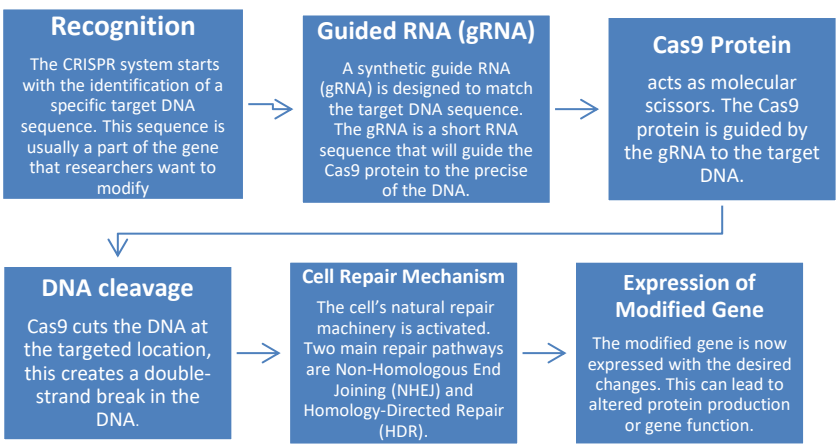

Fig. 1. Step-by-step workings of the CRISPR-Cas9 system

4. CRISPR-Cas SYSTEM'S GROUPS AND CLASSES

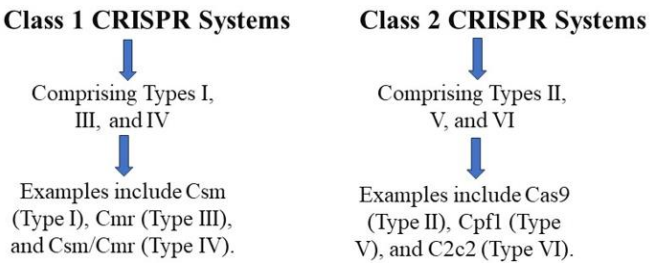

Fig. 2. Types of CRISPR Systems

CRISPR-Cas systems can be broadly categorized into two main classes—Class 1 (I, III, IV) and Class 2 (II, V, VI)—which further divide into six groups. Class 1 systems involve CRISPR ribonucleoproteins as effector nucleases, utilizing more than one Cas protein. Notably, approximately 90% of all CRISPR-Cas genes fall within Class 1, and these systems are found in both bacteria and archaea.

On the other hand, Class 2 systems (II, V, VI) operate with a single Cas protein and are exclusive to bacteria, constituting about 10% of all CRISPR-Cas systems. In Class 2 systems, the CRISPR effector nucleases form ribonucleoproteins, combining crRNA and Cas proteins. The crRNA guides the system to specific locations on the genome, facilitated by the presence of a matching PAM (protospacer adjacent motif) sequence on the target DNA. When crRNA and the target DNA share the same PAM sequence, multidomain effector proteins are activated. These ribonucleoprotein complexes enhance the precision of genome editing by incorporating modified crRNA, thereby improving the accuracy of the guide sequence. Overall, the diverse classes and types within the CRISPR-Cas systems highlight the complexity of these molecular tools, offering researchers a rich landscape to explore and refine for various applications in genetic manipulation.

5. USES

CRISPR promises to revolutionize personalized medicine by tackling genetic mutations implicated in various diseases. Notably, the integration of CRISPR/Cas9 with next-generation sequencing (NGS) is applied in tailoring treatments for cancer [7]. In this application, CRISPR/Cas9 is used to either deactivate or correct genes linked to cancer progression, while NGS identifies patient-specific genetic mutations. The genetic information gathered serves as the foundation for crafting personalized treatment strategies, aiming to precisely address the distinct genetic abnormalities found in the cancer cells of individual patients [8].

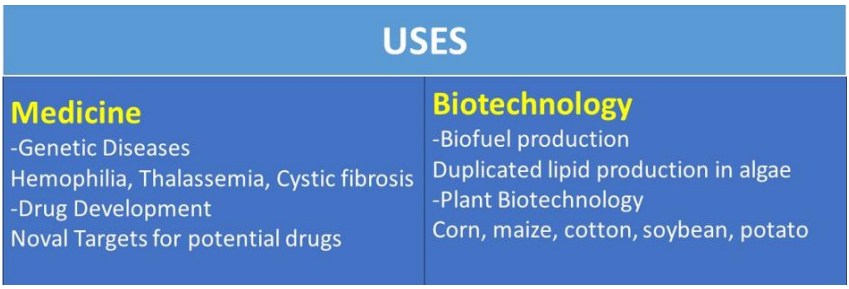

Fig. 3. Uses of CRISPR

An emerging and promising approach is the use of saturation genome editing, employing CRISPR to systematically alter each nucleotide in a gene, enabling the differentiation between harmless and harmful mutations. Furthermore, CRISPR-based techniques, such as the modification of T cells for CAR-T cancer therapy and the treatment of inherited blindness, exemplify CRISPR's potential in advancing the field of precision medicine [9]. These diverse applications underscore the versatility of CRISPR and its role in pushing the boundaries of precision medical treatments.

6. ADVANCEMENT IN CRISPR

The field of CRISPR genome editing is continually advancing, with recent discoveries and developments contributing to its progress. The Nm2Cas9 system, a newly developed type-II Cas9 ortholog, has garnered attention due to its compact effector protein size and simple protospacer adjacent motif (PAM) requirement, making it a promising alternative for genome engineering and gene therapy [10].

Additionally, various Cas12a orthologs have demonstrated editing capabilities in human cells, with BhCas12b being engineered as a powerful gene editing tool [11,12]. New subtypes of the type-V CRISPR system, such as Cas12g, Cas12h, and Cas12i, have been identified from metagenomes, exhibiting unique characteristics and serving as programmable endonucleases for cleaving single-stranded DNA (ssDNA), single-stranded RNA (ssRNA), or double-stranded DNA (dsDNA) in vitro [13].

CasX, now classified into the Cas12e family, has been repurposed as an effective genome editing tool in human cells, while Cas14, belonging to Cas12f, has shown genome editing potential in human cells, albeit with low efficiency. Notably, Cas12k was identified as an RNA-guided site-specific integration system in E. coli, suggesting the potential for CRISPR tools to achieve precisely targeted DNA insertion in the mammalian genome [14].

Beyond the Class-II CRISPR systems, the Class-I CRISPR system with multiple effectors has been utilized to engineer the human genome through diverse strategies. This includes using native nuclease effectors for cleavage or employing fused FokI. Notably, certain members of type-I F systems have been repurposed as tools for site-specific DNA integration. These discoveries underscore the dynamic nature of CRISPR research, with implications for advancing technology and inspiring further exploration in CRISPR biology [15].

Several recent advancements in CRISPR technology have likely occurred. Here are some recent advancements:

A. Prime Editing: Developed as a more precise and versatile genome editing technique, Prime Editing enables the insertion, deletion, or substitution of specific DNA sequences without causing double-strand breaks [16].
B. CRISPR Base Editing: An extension of CRISPR-Cas9, base editing allows for the direct, irreversible conversion of one DNA base pair into another without creating double-strand breaks [17].
C. CRISPR Screens and Functional Genomics: Advancements in CRISPR screening techniques have facilitated large-scale functional genomics studies, enabling the identification of genes associated with specific biological functions or diseases [18].
D. CRISPR Therapeutics: Progress in using CRISPR for therapeutic purposes, including ongoing clinical trials for diseases like sickle cell anemia and beta-thalassemia [19].
E. Enhanced CRISPR Delivery Systems: Development of improved delivery methods for CRISPR components, including nanoparticles and viral vectors, to enhance precision and efficiency in genome editing applications [20].
F. CRISPR Imaging and Tracking: Utilizing CRISPR components for real-time imaging and tracking of specific genomic loci or RNA molecules within living cells [21].

7. CONCLUSION

CRISPR-Cas technology has brought about a significant transformation in biology, enabling swift gene modifications. While the molecular tools associated with CRISPR hold immense potential, their current utility is somewhat limited due to their dependency on specific target cells or DNA systems. Researchers find themselves navigating the challenges of working with or without templates, as the body's natural DNA repair mechanisms can rectify breaks induced by Cas9 and Cas12a-based technologies. However, the effectiveness of these methods varies across different cell types, presenting a hurdle. CRISPR can place DNA precisely without relying on the host cell's DNA repair mechanisms. The ongoing efforts to enhance this technology aim to achieve more accurate and reliable gene modifications by precisely incorporating DNA into target locations within the gene.

In conclusion, the field is actively engaged in refining CRISPR technology, driven by the goal of achieving greater precision in gene editing. The exploration of CRISPR-associated transposase and the continuous improvement of techniques underscore the commitment to advancing this revolutionary tool. The journey towards more effective and reliable gene manipulation techniques remains an ongoing endeavor, with researchers diligently working to unlock the full potential of CRISPR technology.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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Chapter 11
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Impact of Phytase and Cholecalciferol Supplementation on the True Digestibility of Phosphorus by Broiler Chickens Fed Some Agro-Based Byproducts

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ABSTRACT

This research is aimed to determine the effects of phytase and vitamin D_3 supplementation in Cottonseed Meal (CM) and Rice Husk (RH)-based diets on true phosphorus digestibility (TPD) by broiler chickens (BC). Two studies were conducted with 576 one-day-old broiler chickens using regression analysis to determine the TPD in these diets and the response to phytase supplementation. Six semi-purified diets were formulated to contain 150g, 300g, and 450g each of CM/kg (experiment 1) and RH/kg (experiment 2) with phytase supplied at 0 and 1000 units/kg. A total of 288 BC in each study were allotted to the six diets with six replicates of eight birds in a randomized complete block design. In another study (experiment 3), 125 BC were allotted to five treatments comprising a Positive Control (PC, D1) without CM and four CM-based semi-purified diets (31.5%): Negative Control (NC, D2), NC+vitaminD_3 (D3), NC+phytase (D4) and NC+phytase+vitamin D_3 (T5) replicated five times. Similarly, 125 BC were randomly allotted to five treatments comprising a PC (D1) without RH and four RH-based semi-purified diets (30%): NC (D2), NC+ vitaminD_3 (D3), NC+phytase (D4) and NC+phytase+vitaminD_3 (D5). All birds were fed the experimental diets from day 21 to day 28 post-hatch. True phosphorus digestibility was 0.82 for CM

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and 0.75 for RH without phytase; and 0.95 for CM and 0.92 for RH with phytase. Phytase supplementation resulted in 13.27 and 17.94% increases in TPD; and 12.29 and 13.61% increases in TPR by birds fed the CM and RH diets, respectively. In experiment 3 (CM-based diet), ileal phosphorus significantly ($P<0.05$) reduced (0.08) with a corresponding increase in the percentage apparent phosphorus digestibility (90. 64) in D3. There was a significant ($P<0.05$) reduction in excreta phosphorus and a corresponding increase in the percentage of apparent phosphorus retention in the D5. In the RH-based diets, Digestible phosphorus was significantly ($P<0.05$) improved in D3. Excreta phosphorus was significantly reduced in the supplemented diets. Phytase and Vit D_3 supplementation of CM and RH-based diets increased TPD, Digestible P and improved TPR and true ileal phosphorus digestibility in broiler chickens.

Keywords: Arbor acre; ileal phosphorus digestibility; cholecalciferol; semi-purified diet; apparent phosphorus digestibility.

1. INTRODUCTION

Feedstuffs of plant origin contain anti-nutritional factors such as phytic acid (PA) and non-starch polysaccharides (NSP), which limit nutrient utilization in poultry. Phytic acid contains phosphorus, which is poorly digested by poultry, and has the capacity to bind to and reduce the utilisation of other nutrients, whereas NSP are indigestible and have the capacity to reduce nutrient utilisation by encapsulation [1]. Phosphorus (P) is an indispensable nutrient for plants and animals but has limited bioavailability in plant-derived feed ingredients for poultry. Most of the P in grains and oilseed meals is in the form of phytate [2]. Phytate is the principal storage form of phosphorus in plants and it chelates nutrients – minerals such as potassium, magnesium, and calcium – which are necessary for phosphorus absorption [3]. Phytate is poorly utilized by poultry because they have limited amounts of intestinal phytase. As a result, poultry diets are supplemented with inorganic P sources, resulting in large amounts of P in the diet, which are subsequently passed into the environment, causing eutrophication [4]. The activities of intrinsic phytase in diets for poultry and endogenous phytase in the digestive tract are not enough for efficient hydrolysis of phytate [3,5]. Dietary supplementation of phosphorus with exogenous phytase is effective in improving phosphorus digestion in poultry diets [6]. But supplementing poultry diets with phosphorus based on feed formulation calculations may lead to a deficiency in phosphorus or the excretion of excess dietary phosphorus into the environment [7]. Rodehutscord [8] asserted that in assessing P availability, the measurement of digestible P remains the preferred method for poultry. The shift from measuring apparent digestible P to True digestible phosphorus (TDP) of ingredients used in feeding poultry occurs because of varying proportions of phytate and the limitations of measurements of apparent digestibility. The use of TPD provides a more reliable template for measuring digestible P in broiler diets.
Apparent P digestibility estimates do not account for the confounding effect of endogenous P losses (EPL). The estimation of EPL of some feed ingredients by the regression technique is important to determine the TPD of ingredients in broiler feed [7]. Endogenous phosphorus loss is reduced and hence TPD may

increase with phytase supplementation owing to the increased release of P from the phytate-phosphorus complex [9,7]. Ample research findings are available on TPD in feed ingredients used in pigs (Kriseldi et al., 2021); [9]. Corresponding data for poultry are limited, however, with few research studies [7,10,11].

Commonly, vitamin D3 has been consumed in birds such as chickens, turkeys, ducks, and geese nutrition as a forebear for calcitriol. In recent years, however, vitamin D metabolites such as 25 hydroxycholecalciferol (25-OHD3) have been successfully consumed in birds' nutrition (Stein et al., 2011). Kheiri et al. [12] compared the efficacy of 1-α(OH)D3 alone or in a combination of microbial phytase on broiler quail's performance and quality of tibia; the result indicated positive interaction between 1-α(OH)D3 and phytase on bone mineralization. Shahab et al. [13] reported that supplementation of 1-α(OH)D_3 in combination with phytase and cholecalciferol in Japanese quail diet resulted in better performance.

True digestible phosphorus in some feed ingredients has been documented, but TDP results might differ across climactic regions based on differences in the ingredients, the method of oil extraction, agronomical practice during cultivation, and the composition of the assay diets. There are also a few trials on cholecalciferol on broiler chickens. The objective of this study was to determine TPD in response to phytase supplementation of broiler chickens fed diets containing Cottonseed Meal (CM) and Rice Husk (RH), using the regression technique. To also determine the effect of supplementing low P diets containing CM and RH with vitamin D_3 and phytase on P digestibility and retention in broiler chickens.

2. MATERIALS AND METHODS

The ethics committee on Research and Innovation of the University of Ibadan approved this research on 28 April 2016. This approval conforms to the ethical standards laid down in 1964 Declaration of Helsinki and its later amendment. All birds were healthy throughout the studies and no mortality was recorded.

The test ingredients used in the experiments were CM and RH. Rice husk is a by-product of rice grains that are obtained by mechanical milling in contrast to the solvent extraction technique that is used for CM extraction. In experiments 1 and 2, a total of 576 one-day-old Abor Acre broiler chicks were raised in floor pens in a well-ventilated and illuminated standard poultry house. On arrival, the birds were fed a commercial starter diet, which met the nutrient requirements for broiler chickens for 14 days [14]. On day 14, the birds were transferred to metabolic cages. On day 20 post-hatch, two groups of 288 birds were tagged, weighed individually, and randomly allotted to experimental diets in each of the two experiments, with six replicates in a randomized complete block design using the experimental animal allotment programme of Kim and Lindemann [15]. Birds had free access to water and the experimental diets for eight days, with the first two days being allowed for acclimatization to the experimental diets.

Table 1. Gross composition (as fed) of cottonseed meal-based diets without or with phytase for broiler chickens ingredients

	0 FTU/Kg (without Phytase)			1000FTU/Kg (with Phytase)		
Ingredients	**150CM g/kg**	**300CM g/kg**	**450CM g/kg**	**150CM g/kg**	**300CM g/kg**	**450CM g/kg**
Cottonseed meal	150.00	300.00	450.00	150.00	300.00	450.00
Cassava Starch	495.50	343.75	192.00	485.50	333.75	182.00
Wheat gluten	200.00	200.00	200.00	200.00	200.00	200.00
Soya oil	10.00	10.00	10.00	10.00	10.00	10.00
Dextrose	102.25	102.25	102.25	102.25	102.25	102.25
DL-Methionine	1.00	1.00	1.00	1.00	1.00	1.00
Lysine	1.00	1.00	1.00	1.00	1.00	1.00
Limestone	10.25	12.00	13.75	10.25	12.00	13.75
Limestone	11.25	13.25	15.25	11.25	13.25	15.25
Vitamin-premix[1]	2.50	2.50	2.50	2.50	2.50	2.50
Salt	2.50	2.50	2.50	2.50	2.50	2.50
Phytase premix[2]	0.00	0.00	0.00	10.00	10.00	10.00
Titanium dioxide	25.00	25.00	25.00	25.00	25.00	25.00
Premix[3]						
Total	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

[1]vitamin A: 12500 I.U, vitamin E: 40 mg, vitamin K3: 2 mg, vitamin B1: 3 mg, vitamin B2: 5.5 mg, niacin: 5.5 mg, calcium pantothenate: 11.5 mg, vitamin B6: 5 mg, vitamin B12: 0.025 mg, choline chloride: 500 mg, folic acid:1 mg, biotin: 0.08 mg, manganese: 120 mg, iron 100 mg, zinc: 80 mg, copper: 8.5 mg, iodine: 1.5 mg, cobalt: 0.3 mg, [2]1 g of enzyme mixed with 99g of cassava starch to contain 100 phytase units (FTU)/g, [3]5 g titanium dioxide mixed with 20 g of corn starch, CM: cottonseed meal

Table 2. Estimated nutrient densities in cottonseed meal-based diets for broiler chickens without or with supplemental phytase

	0 FTU/kg(Phytase)			1000FTU/kg(Phytase)		
Nutrient	**150CM, g/kg**	**300 CM, g/kg**	**450 CM, g/kg**	**150 CM, g/kg**	**300 CM, g/kg**	**450 CM, g/kg**
Dry matter, g/kg	965	957	931	930	930	93.5
Crude protein, g/kg	255	276	291	227	264	292
Gross energy, kcal/kg	3940	3880	3880	3950	3920	3700
Calcium, g/kg	3.80	4.66	5.30	4.00	4.17	4.20
Phosphorus, g/kg	1.76	3.75	5.07	1.60	3.18	4.79
Calcium: Phosphorus	2.16	1.24	1.05	2.50	1.31	0.85
Phytase, FTU/kg	<100	<100	<100	854	986	1106

CM: Cottonseed meal

Table 3. Gross composition (as fed) of rice husk-based diets without or with phytase for broiler chickens ingredients

	0 FTU/Kg (without Phytase)			1000FTU/Kg (with Phytase)		
Ingredients	**150RH g/kg**	**300 RH g/kg**	**450 RH g/kg**	**150 RH g/kg**	**300 RH g/kg**	**450 RH g/kg**
Rice Husk	150.00	300.00	450.00	150.00	300.00	450.00
Cassava Starch	464.50	316.25	168.00	454.50	306.25	158.00
Wheat gluten	220.00	220.00	220.00	220.00	220.00	220.00
Soya oil	20.00	16.25	12.50	20.00	16.25	12.50
Dextrose	102.25	102.25	102.25	102.25	102.25	102.25
DL-Methionine	1.00	1.00	1.00	1.00	1.00	1.00
Lysine	1.00	1.00	1.00	1.00	1.00	1.00
Limestone	10.25	12.00	13.75	10.25	12.00	13.75
Limestone	11.25	13.25	15.25	11.25	13.25	15.25
Vitamin-premix[1]	2.50	2.50	2.50	2.50	2.50	2.50
Salt	2.50	2.50	2.50	2.50	2.50	2.50
Phytase premix[2]	0.00	0.00	0.00	10.00	10.00	10.00
Titanium dioxide Premix[3]	25.00	25.00	25.00	25.00	25.00	25.00
Total	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

[1]vitamin A: 12500 I.U, vitamin E: 40 mg, vitamin K3: 2 mg, vitamin B1: 3 mg, vitamin B2: 5.5 mg, niacin: 5.5 mg, calcium pantothenate: 11.5 mg, vitamin B6: 5 mg, vitamin B12: 0.025 mg, choline chloride: 500 mg, folic acid:1 mg, biotin: 0.08 mg, manganese: 120 mg, iron 100 mg, zinc: 80 mg, copper: 8.5 mg, iodine: 1.5 mg, cobalt: 0.3 mg

[2]1 g of enzyme mixed with 99g of cassava starch to contain 100 phytase units (FTU)/g

[3]5 g titanium dioxide mixed with 20 g of cornstarch

RH: rice husk

Table 4. Estimated nutrient densities in rice husk-based diets for broiler chicks without or with supplemental phytase

	0 FTU/kg (Phytase)			1000 FTU/kg (Phytase)		
Nutrient	**150 RH g/kg**	**300 RH g/kg**	**450 RH g/kg**	**150 RH g/kg**	**300 RH g/kg**	**450 RH g/kg**
Dry matter, g/kg	974	970	965	972	976	965
Crude protein, g/kg	185	206	245	191	207	225
Gross energy, kcal/kg	3458	3355	3252	3423	3320	3217
Calcium, g/kg	0.53	0.58	0.62	0.34	0.45	0.53
Phosphorus, g/kg	0.21	0.24	0.30	0.22	0.26	0.32
Calcium: Phosphorus	2.52	2.42	2.07	1.55	1.73	1.66
Phytase, FTU/kg	<100	<100	<100	1131	1007	1247

RH: Rice husks

Six semi-purified diets were formulated containing 150 g, 300 g, and 450 g each of CM and RH/kg (Tables 1 and 2, respectively) with or without 1000 units of phytase/kg diet. The dietary inclusion levels of the test ingredients were obtained by the gradual replacement of cassava starch with the CM or RH for the two experiments, respectively. Titanium dioxide was included in the diets at the rate of 5 g/kg as an indigestible marker. Phytase (3-phytase derived from *Aspergillus niger*) was used as the exogenous phytase.

Table 5. Gross composition (as fed) of cottonseed meal-based diets for broiler chickens fed phytase and vitamin D_3 supplemented diet

	PC	NC	NC+Vit D_3	NC+Phy	NC+Phy+Vit D_3
Ingredients	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
Cottonseed meal	0.00	315.00	315.00	315.00	315.00
Cassava Starch	608.00	469.00	468.75	459.00	458.75
Wheat gluten	250.00	100.00	100.00	100.00	100.00
Soya oil	10.00	2.00	2.00	2.00	2.00
Dextrose	90.00	90.00	90.00	90.00	90.00
Methionine	1.00	1.00	1.00	1.00	1.00
Lysine	1.00	1.00	1.00	1.00	1.00
Limestone	17.00	12.00	12.00	12.00	12.00
Vitamin-Premix	2.50	2.50	2.50	2.50	2.50
Salt	2.50	2.50	2.50	2.50	2.50
Phytase (FTU/kg)	0.00	0.00	0.00	10.00	10.00
Cholecalciferol (g/kg)	0.00	0.00	0.25	0.00	0.25
Titanium dioxide premix	5.00	5.00	5.00	5.00	5.00
Dicalcium phosphate	13.00	0.00	0.00	0.00	0.00
Total	1000.00	1000.00	1000.00	1000.00	1000.00
Calculated Nutrients					
ME Kcal/Kg	3575.37	3158.31	3157.44	3575.37	3575.37
CP (g/kg)	218.00	217.61	217.61	217.61	217.61
Ca (g/kg)	10.85	5.64	5.64	5.64	5.64
Total P (g/Kg)	4.84	4.06	4.06	4.06	4.06
Non Phytate P (g/kg)	4.84	1.69	1.69	1.69	1.69
Phytate P (g/kg)	0.00	2.36	2.36	2.36	2.36
Ca : NPP ratio	2.24	3.33	3.33	3.33	3.33
Ca : P ratio	2.24	1.39	1.39	1.39	1.17

[1]Composition of vitamin premix per kg of diet: vitamin A, 12500 I.U; vitamin E, 40mg; vitamin K_3, 2mg; vitamin B_1, 3mg; vitamin B_2, 5.5mg; niacin, 5.5mg; calcium pantothenate, 11.5mg; vitamin B_6, 5mg; vitamin B_{12}, 0.025mg; choline chloride, 500mg, folic acid,1mg; biotin, 0.08mg; manganese, 120mg; iron 100mg; zinc, 80mg; copper, 8.5mg; iodine, 1.5mg; cobalt, 0.3mg; selenium, 0.12mg, anti-oxidant, 120mg, [2]Phytase premix prepared by mixing phytase with maize. [3]Titanium dioxide premix prepared by mixing 1g of titanium oxide with 4g of maize

In experiment 3, at 20-day-old post-hatch, 250 broiler chickens were randomly allotted to five semi-purified diets containing CM and RH as the test ingredient.

For the CM diets, a Positive Control (PC) diet with required non-phytate phosphorus (NPP) (4.84 g/kg diet) and a Negative Control (NC) diet in which the NPP is reduced by 65% (i.e. from 4.84 to 1.69 g/kg diet) was formulated. While for the RH diet, a Positive Control (PC) diet with required NPP (4.84 g/kg diet) and a Negative Control (NC) diet in which the NPP is reduced by 45% (i.e. from 4.84 to 2.66 g/kg diet) was formulated. Three other diets each for CM and RH in which the NC diet is supplemented with either 1000 FTU phytase/kg diet, or 10,000 IU. vitamin D_3/kg diet or both was formulated, making a total of 10 semi-purified diets. On day 21, the chicks were individually weighed and allocated to the 10 diets in a randomized complete block design with 5 replicates of 5 birds each. Five cages were randomly assigned to each of the 5 treatments (CM and RH respectively).

Table 6. Estimated nutrient densities in cottonseed meal-based diets (g/100 g) for broiler chicks fed low P diets supplemented with Phytase or Vit D_3

Nutrient	PC T1	NC T2	NC+Vit D_3 T3	NC+Phy T4	NC+Phy+Vit D_3 T5
Dry Matter	916	908.5	906.1	912.7	903.9
Crude Protein	205.5	192.5	218.0	203.5	201.0
Calcium	5.20	3.36	4.24	4.09	4.27
Total P	0.77	0.87	0.80	0.75	1.09
Gross energy (Kcal/kg)	3256	3112	3122	3197	3322

CM: Cottonseed meal

Table 7. Gross composition (as fed) of rice husk-based diets for broiler chickens fed phytase and vit D_3 supplemented diet

	PC	NC	NC+VitD_3	NC+ Phy	NC+Phy+ Vit D_3
Ingredients	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
Rice Husk`(RH)	0.00	300.00	300.00	300.00	300.00
Cassava Starch	608.00	378.00	377.75	368.00	367.75
Wheat gluten	250.00	200.00	200.00	200.00	200.00
Soya oil	10.00	2.00	2.00	2.00	2.00
Dextrose	90.00	90.00	90.00	90.00	90.00
Methionine	1.00	1.00	1.00	1.00	1.00
Lysine	1.00	1.00	1.00	1.00	1.00
Limestone	17.00	18.00	18.00	18.00	18.00
Vitamin-Premix	2.50	2.50	2.50	2.50	2.50
Salt	2.50	2.50	2.50	2.50	2.50
Phytase (FTU/kg)	0.00	0.00	0.00	10.00	10.00
Cholecalciferol (g/kg)	0.00	0.00	0.25	0.00	0.25
Titanium dioxide premix	5.00	5.00	5.00	5.00	5.00
Dicalcium phosphate	13.00	0.00	0.00	0.00	0.00

	PC	NC	NC+VitD_3	NC+ Phy	NC+Phy+ Vit D_3
Ingredients	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
Total	1000.00	1000.00	1000.00	1000.00	1000.00
Calculated Nutrients					
ME Kcal/Kg	3575.37	3391.22	3390.35	3356.32	3355.44
CP (g/kg)	218.00	213.10	213.10	213.10	213.10
Ca (g/kg)	10.85	8.27	8.27	8.27	8.27
Total P (g/Kg)	4.84	6.50	6.50	6.50	6.50
NPP (g/kg)	4.84	2.66	2.66	2.66	2.66
Phytate P (g/kg)	0.00	3.84	3.84	3.84	3.84
Ca : NPP ratio	2.24	3.11	3.11	3.11	3.11
Ca : P ratio	2.24	1.27	1.27	1.27	1.27

[1]Composition of vitamin premix per kg of diet: vitamin A, 12500 I.U; vitamin E, 40 mg; vitamin K_3, 2 mg; vitamin B_1, 3 mg; vitamin B_2, 5.5 mg; niacin, 5.5 mg; calcium pantothenate, 11.5 mg; vitamin B_6, 5 mg; vitamin B_{12}, 0.025 mg; choline chloride, 500 mg, folic acid,1mg; biotin, 0.08 mg; manganese, 120 mg; iron 100mg; zinc, 80 mg; copper, 8.5 mg; iodine, 1.5 mg; cobalt, 0.3 mg; selenium, 0.12 mg, anti-oxidant, 120 mg, [2]Phytase premix prepared by mixing phytase with maize. [3]Titanium dioxide premix prepared by mixing 1 g of titanium oxide with 4 g of maize

Table 8. Estimated nutrient densities in rice husk-based diets (g/100g) for broiler chicks fed low P diets supplemented with Phytase or Vit D_3

Components (g/kg)	PC D1	NC D2	NC+Vit D_3 D3	NC+Phy D4	NC+Phy+Vit D_3 D5
Dry Matter	905.4	910.9	906.9	908	912.6
Crude Protein	200.7	200.1	200.5	213.0	205.5
Calcium	8.19	6.05	8.19	5.49	6.20
Total P	1.83	1.76	1.79	1.90	1.77
Gross energy (Kcal/kg)	3456	3312	3321	3212	3235

RH: Rice husks

The CM used in this current study contained more CP and total P, but less GE and Ca than RH (Table 5). Increases in the contents of CP, Ca and total P in the diets without or with phytase were because of the graded inclusion of CM and RH at the expense of cassava starch in the two experiments.

Nutrient densities for the CM and RH diets are presented in Tables 6 to 9, respectively. In general, the diets that were based on RH were less nutrient dense than those based on CM.

Feed intake was calculated as the difference between amounts offered and refused on a cage basis during the eight-day feeding trial. Birds were weighed at days 20 and 28 post-hatch on a cage basis to calculate bodyweight gain. Between days 25 and 27 post-hatch, samples of fresh excreta were collected once daily from pans placed beneath each cage. Daily collections were pooled

on a replicate cage basis, bulked, and stored in the freezer at -4°C. Samples were taken and air-dried in a force-draught oven at 55 °C for five days. On day 28, the birds were euthanized by carbon (IV) oxide asphyxiation and dissected to obtain digesta from the distal two-thirds of the ileum using the procedure of Rodehutscord et al. [16]. Digesta samples were obtained by flushing the ileal content with deionized water, being careful not to squeeze the dissected portion. Samples from each replicate cage were pooled. The digesta samples were frozen and freeze-dried. The excreta and ileal samples were milled and stored in air-tight plastic sample bags at -4 °C until needed for analysis.

Table 9. Analyzed nutrient composition of cottonseed meal and rice husk used in the diets that were fed in this study

Nutrient	Cottonseed meal	Rice husk
Dry matter, g/kg	990	961
Crude protein, g/kg	334	227
Gross energy, kcal/kg	2200	2980
Calcium, g/kg	1.30	5.30
Total phosphorus, g/kg	7.75	4.90
Phytate phosphorus, g/kg	4.50	2.80
Non-phytate Phosphorus, g/kg	3.25	2.10

Dry matter was determined by drying the excreta and ileal samples at 105 °C for 24 hours in a pre-weighed dried crucible in a conventional oven (method no 930.15) [17]. Samples were ashed and phosphorus concentration was determined calorimetrically (UV) at 400 nm following digestion with nitric and perchloric acid. Titanium concentrations in the ashed samples of feed, excreta, and ileal were determined by the colorimetric method following digestion with concentrated tetraoxosulphate (vi) acid and absorbance read at 410 nm [18].

Apparent pre-caecal digestibility and retention coefficients of P in the two experiments were calculated by the index method [7], which is based on indigestible marker ratios.

The dietary P intake was calculated as concentration in grams of P intake per kilogram of DM. The amount of digestible P was calculated from the intake of P and its digestibility, Apparent P retention (APR) was calculated as a proportion of the P intake that was not voided in the excreta.

Data were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, North Carolina, USA). Each experiment was analyzed separately. The model consisted of replications (5 df), the level of the basal ingredient (2 df), phytase level (1 df), and the interaction (2 df). Orthogonal polynomial contrasts were used to determine the effects of graded levels of the basal ingredient on all response criteria. Levels of digested P (g/kg DMI) and retained P (g/kg of DMI) were regressed on P intake (g/kg DM) separately for the two levels of phytase supplementation. Estimates of the regression parameters were compared within sampling sites (ileal and excreta) with or without phytase using student's t-test.

3. RESULTS AND DISCUSSION

With CM as the basal diet, the interaction of its inclusion level with the level of phytase supplementation was significant for ileal output of P (P =0.002), excreted P (P =0.009), APD (P =0.034), but not for APR (P = 0.093). Without phytase, the ileal output of P was greater for birds fed the 300 g/kg CM diet than for birds fed the diets with either a lower or higher level of CM. With phytase, no such effect was observed. Without phytase, the responses in excreted P were somewhat similar to those for ileal output of P, except that the levels of excreted P for birds fed 300 g/kg and 450 g/kg CM were similar. In contrast, when the diets were supplemented with phytase, the excreted P was reduced for birds fed the 300 g/kg CM diet than those fed either the 150 g/kg or 450 g/kg CM diets, which produced similar responses. As a consequence of these effects, the APD was lower for birds fed the intermediate level of CM compared to either extreme when phytase was not provided. However, when phytase was provided there was a graded increase in APD with the level of CM that was fed. Either with or without phytase supplementation, there was a marked increase in APR from the low level of dietary CM to the higher levels which were nearly similar to each other. The effect of phytase supplementation on APR was negligible (P =0.35).

Far fewer significant effects were observed with the RH based diets than were observed when graded levels of CM were fed (Table 10). Interactions between the level of dietary RH and supplemental phytase were not significant for ileal P output, excreted, APD or APR (P ≥0.5). Supplementation with phytase increased (P =0.04) APR across all levels of RH that were fed.

There were no linear and quadratic responses (P >0.05) in the ileal output and apparent ileal P digestibility of birds on the RH-based diet, irrespective of supplementation. A quadratic response was observed in excreta P output (P <0.01) at 300 g/kg RH inclusion for diets supplemented with phytase. Apparent ileal digestibility values ranged between 88.52% and 92.30% and 93.34% to 93.53% for diets without supplemental phytase and with supplemental phytase, respectively. Apparent ileal phosphorus digestibility for birds fed phytase-supplemented diets was 9.32% higher than the value calculated for birds fed unsupplemented diets. Apparent P retention values ranged from 88.26% to 98.25%. Without phytase, apparent P retention did not differ (P >0.05), but with the supplementation of phytase there was a quadratic response (P <0.05) at 300 g/kg RH inclusion level. The TPD of RH without phytase at 75% was lower (P <0.01) than the TPD at 92% with phytase supplementation. The true P retention at 91% with phytase supplementation was higher (P <0.01) than that at 78% without phytase (Table 11). Phytase supplementation effectively increased digestible P at the ileal site by 17.94 percentage points and a corresponding increase in true P retention by 13.61 percentage points with the addition of phytase.

Table 10. Phosphorus digestibility in broiler chickens fed varying levels of cottonseed meal without or with phytase

	Without phytase			With phytase			
	150 g/kg	**300 g/kg**	**450 g/kg**	**150 g/kg**	**300 g/kg**	**450 g/kg**	
	CM	**CM**	**CM**	**CM**	**CM**	**CM**	**SEM**
Ileal P output, g/Kg DMI	0.40	1.25	0.98	0.24	0.35	0.40	0.08
Excreted P g/Kg of DMI	0.92	1.49	1.51	1.06	0.69	1.03	0.07
Apparent ileal P digestibility, %	78.19	67.97	81.92	85.97	89.69	92.27	1.80
Apparent P retention, %	49.39	61.95	72.18	38.52	79.90	80.00	3.58

CM: Cottonseed meal P: Phosphorus, DMI: dry matter intake

Table 11. Phosphorus digestibility in broiler chickens fed cottonseed meal and rice husk-based diets without or with phytase

	Without phytase			With phytase			
	150 g/kg	**300 g/kg**	**450 g/kg**	**150 g/kg**	**300 g/kg**	**450 g/kg**	
	RH	**RH**	**RH**	**RH**	**RH**	**RH**	**SEM**
Ileal P output, g/Kg of DMI	0.21	0.19	0.36	0.15	0.17	0.22	0.02
Excreta P output, g/Kg of	0.26	0.25	0.25	0.08	0.24	0.06	0.03
Apparent ileal P digestibility %	90.25	92.30	88.52	93.52	93.53	93.34	0.86
Apparent P retention %	88.26	89.80	89.80	96.67	91.05	98.25	1.46

RH: Rice husks, P: Phosphorus, DMI: dry matter intake

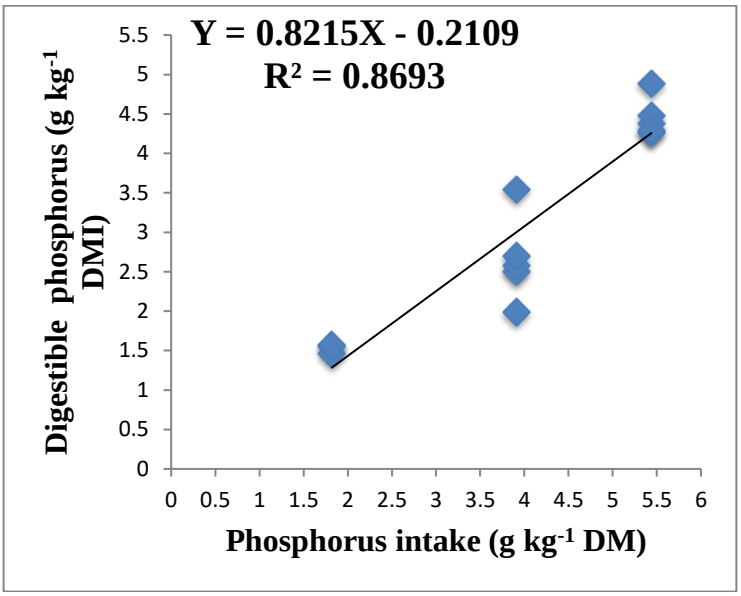

Fig. 1. A linear relationship between digested phosphorus and dietary phosphorus intake at the ileal section of experimental chicks fed cottonseed meal without Natuphos® phytase supplementation

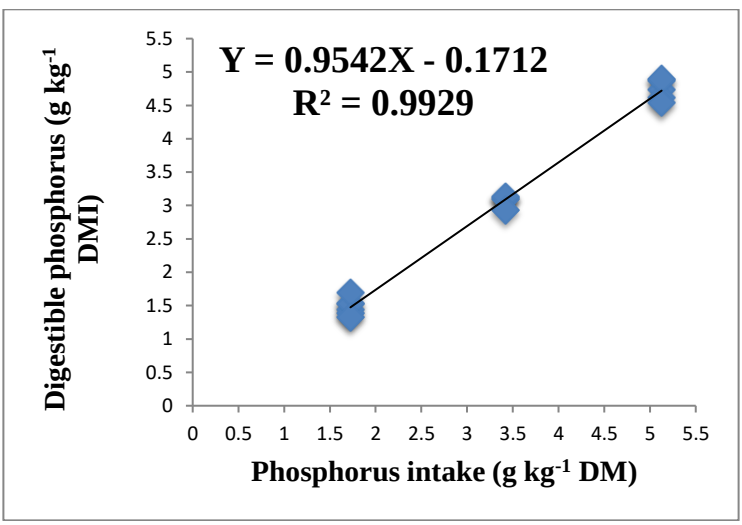

Fig. 2. A linear relationship between digested phosphorus and dietary phosphorus intake at the ileal section of experimental chicks fed cottonseed meal with Natuphos® phytase supplementation

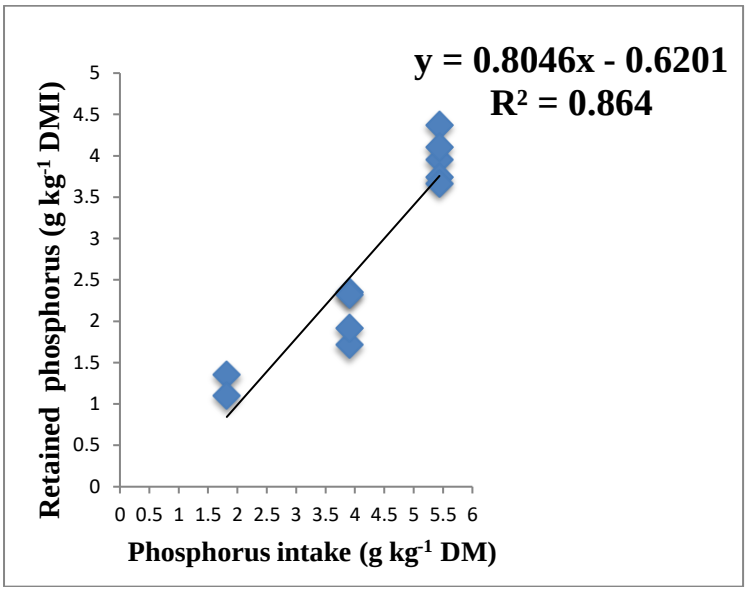

Fig. 3. Linear relationship between retained phosphorus and dietary phosphorus intake at the total tract section of experimental chicks fed cottonseed meal without Natuphos® phytase supplementation

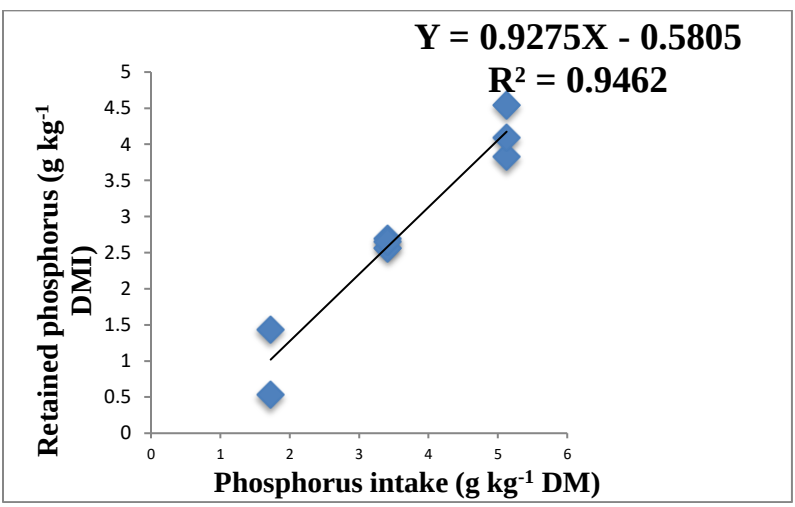

Fig. 4. Linear relationship between retained phosphorus and dietary phosphorus intake at the total tract section of experimental chicks fed cottonseed meal with Natuphos® phytase supplementation

Figs. 1 and 2 are graphs showing the linear relationship between ileal digested phosphorus expressed as g/kg DM intake against phosphorus intake g/kg DM for CSM diets without or with phytase respectively. For CSM diets without phytase, the slope of the graph, which represents the True Ileal Phosphorus Digestibility (TIPD) coefficient was 0.822 with a corresponding EPL value of 0.211g/kg DMI on extrapolation of the regression line to zero P-intake. For phytase supplemented CSM, TIPD coefficient was 0.954, and the corresponding endogenous P loss of 0.171g/kg DMI of CSM. Figs. 3 and 4 depict the linear relationship between retained P (g/kg DMI) against dietary P intake (g/kg DM) for birds fed CSM-based diets without or with phytase respectively.

The True Total Tract Phosphorus Retention (TTTPR) coefficients were 0.805 and 0.928 for CSM without and with phytase respectively, while excreta endogenous phosphorus flows were 0.62 and 0.58g/kg DMI for phytase un-supplemented and supplemented diets respectively.

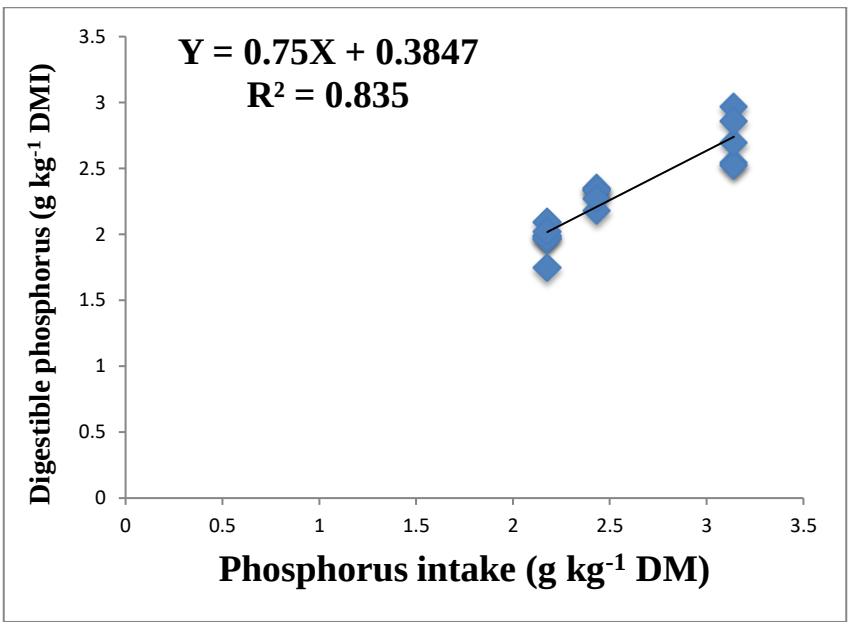

Fig. 5. Linear relationship between digested phosphorus and dietary phosphorus intake at the ileal section of 28-day-old broiler chicks fed rice husk diet without phytase supplementation

Figs. 5 and 6 are graphs showing linear relationship between ileal digested phosphorus against phosphorus intake for RH diets without or with phytase respectively. For RH diets without phytase, the slope of the graph which represents the True Ileal Phosphorus Digestibility (TIPD) coefficient was 0.75 with a corresponding EPL value of 0.385g/kg DMI on extrapolation of the regression line to zero P-intake. For phytase supplemented RH, TIPD coefficient was 0.929 and corresponding endogenous P loss of 0.014g/kg DMI of RH. Figs. 7 and 8 depict the linear relationship between retained P against dietary P intake for birds fed RH-based diets without or with phytase respectively. The True Total Tract Phosphorus Retention (TTTPR) coefficients were 0.782 and 0.918 for RH without and with phytase respectively, while total tract endogenous phosphorus flows were 0.766 and 0.338 g/kg DMI for phytase un-supplemented and supplemented diets respectively.

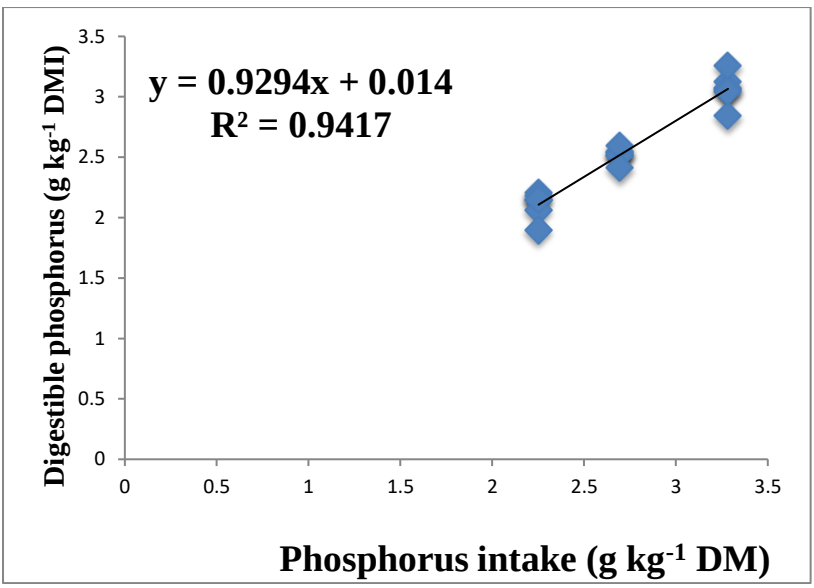

Fig. 6. Linear relationship between digested phosphorus and dietary phosphorus intake at the ileal section of 28-day-old broiler chicks fed rice husk with phytase supplementation

In the context of the current studies, the strong linear relationships that were observed between ileal digested P, retained P, and dietary P intake for all the test ingredients were the primary requirements for the use of the regression technique. Other authors reported a strong linear relationship between digested P outputs and dietary P intake [7,11]. This relationship permits the theoretical estimation of dietary-independent endogenous P loss (g/kg DMI) and the simultaneous measurement of true ileal P digestibility [6]. Estimating the TPD of CM and RH is important because these feedstuffs are used as alternative protein and carbohydrate sources in broiler chicken feed. This result agrees with other

studies, which used soybean meal [6,19,20], canola meal, and peanut flour [7] as the test ingredients. In the diet formulation for CM and RH studies, an increase of P in the diets was achieved by raising the amounts of CM and RH to ensure graded levels of P intake by the birds. This study also involved the development of a database of TPD values for a wide range of feedstuffs. Up-to-date knowledge of the effect of microbial phytase on TPD would assist in formulating diets that would minimize P excretion. The range in phytase activity in the unsupplemented diets was less than 100 units/kg, whereas the diets with supplemental phytase activity ranged from 854 to 1247 units/kg. Weremko et al. [21] reported phytase activity above 500 units/kg to increase P digestibility but less distinctly, but a further rise in phytase dosage (up to 1000 units) increased P digestibility.

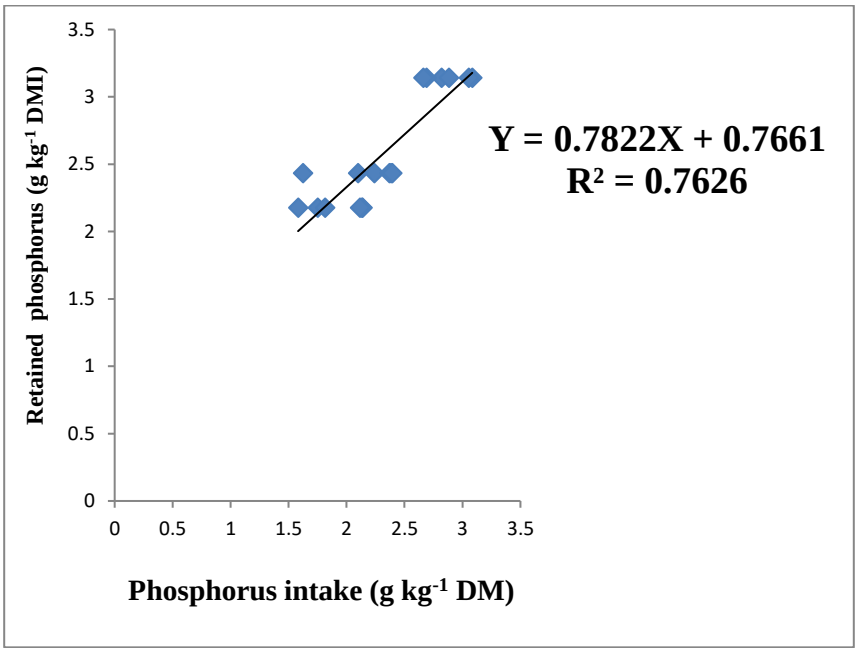

Fig. 7. Linear relationship between retained phosphorus and dietary phosphorus intake at total tract section of 28-day-old experimental chicks fed rice husk without natuphos® phytase supplementation

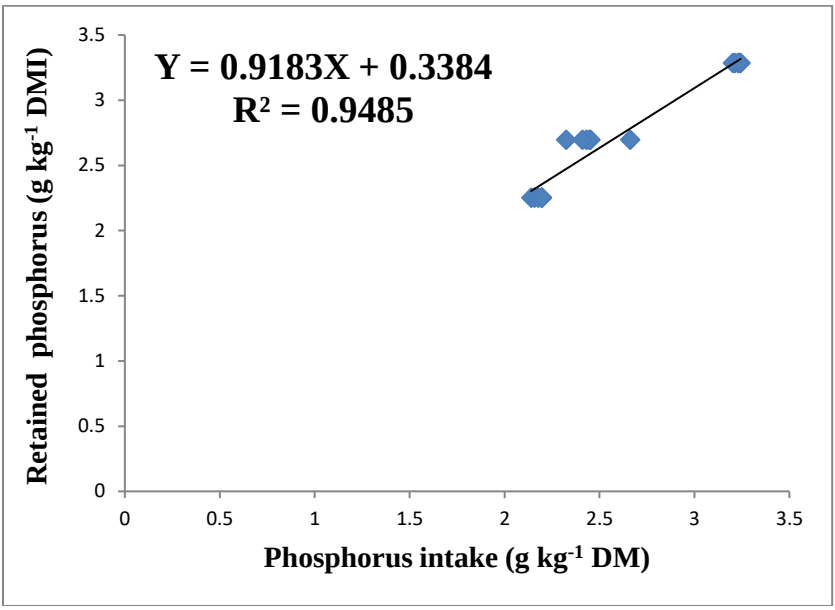

Fig. 8. Linear relationship between digested phosphorus and dietary phosphorus intake at total tract section of 28-day-old broiler chicks fed rice husk with natuphos® phytase supplementation

Ileal phosphorus significantly (P<0.05) reduced with a corresponding increase in the percentage apparent phosphorus digestibility in the diet supplemented with vitamin D_3 (D3), while D4 and D5 did not differ from D1 in the two parameters. Digestible P was significantly improved for birds on D5 in comparison to the other four diets. Digestible P in D4 did not differ (P>0.05) from the birds on the PC (D1) diet. There was a significant reduction in excreta phosphorus and a corresponding increase in the percentage apparent phosphorus retention in the PC and the diets supplemented with a combination of phytase and vitamin D_3 (D5) when compared to the NC (D2) diets, D3 and D4. Phosphorus retention improved in D5 which compares (P>0.05) favourably with the PC (D1) diets. While D3 and D4 did not differ significantly from the NC diets for both phosphorus retention and percentage apparent phosphorus retention.

There was no significant (P>0.05) difference in the percentage bone ash, ileal, retained and percentage apparent phosphorus retention across all the treatments. Digestible phosphorus in D3 was significantly (P<0.05) improved when compared to the PC (D1) diet. Whereas there was no (P>0.05) significant improvement between D1, D4, and D5. Excreta phosphorus was significantly reduced in the supplemented diets (D3, D4, and D5) and the PC (D1) when compared with the NC diets.

Table 12. Linear relationships of ileal digestible intake phosphorus and retained phosphorus with phosphorus intake of broiler chickens fed cottonseed meal or rice husk-based diets without and with phytase supplementation

Item	Regression of ileal digestible phosphorus on phosphorus intake		Regression of retained phosphorus on phosphorus intake	
	Without phytase	With phytase	Without phytase	With phytase
Cottonseed meal augmented diets				
Slope	0.821	0.954	0.805	0.928
Intercept	0.408	0.081	0.471	0.350
R^2	0.869	0.993	0.864	0.946
Endogenous P lost, g/kg DMI	0.211	0.171	0.620	0.581
True ileal P digestibility or true P retention, %	82[b]	95[a]	80[a]	93[b]
Rice husk augmented diets				
Slope	0.750	0.929	0.782	0.918
Intercept	0.231	0.161	0.356	0.167
R^2	0.835	0.942	0.763	0.949
Endogenous P loss, g/kg DMI	0.385	0.014	0.766	0.338
True ileal P digestibility or true P retention, %	75[b]	92[a]	78[b]	91[a]

[a,b] Estimates with a common superscript were not different at P = 0.05

Table 13. Phosphorus intake, phosphorus outputs, and calculated phosphorus response criteria of 28-day-old broilers[1] fed cottonseed meal-based diets

Measurements	PC D1	NC D2	NC+Vit D_3 D3	NC+Phy D4	NC+Phy+Vit D_3 D5	SEM
Ileal phosphorus (g/kg DMI)	0.20^{ab}	0.26^{a}	0.05^{c}	0.08^{bc}	0.11^{bc}	0.25
Apparent phosphorus Digestibility (%)	76.61^{b}	75.81^{b}	94.61^{a}	90.64^{ab}	90.11^{ab}	2.53
Excreta phosphorus (g/kg DMI)	0.20^{c}	0.78^{a}	0.55^{b}	0.56^{b}	0.51^{b}	0.04
Apparent phosphorus retention (%)	76.99^{a}	26.18^{c}	37.67^{c}	35.63^{c}	54.38^{b}	4.00

$^{a\,b\,c}$ Means in a row with different superscripts are significantly different from each other (P<0.05)
[1]Each value represents mean of 5 replicates (5 birds/replicate), PC= Positive control, NC: Negative control
[2]Pooled standard error of mean, DM: Dietary dry matter content, DMI: dry matter Intake

Table 14. Phosphorus intake, phosphorus outputs and calculated phosphorus response criteria of 28-day-old broilers fed rice husk-based diets

Measurements	**PC D1**	**NC D2**	**NC+Vit D_3 D3**	**NC+Phy D4**	**NC+Phy+Vit D_3 D5**	**[2]SEM**
Ileal phosphorus (g/kg DMI)	0.11	0.13	0.14	0.14	0.21	0.02
Apparent phosphorus Digestibility (%)	80.78^{ab}	89.53^{a}	81.88^{ab}	75.97^{ab}	67.45^{b}	2.82
Excreta phosphorus (g/kg DMI)	0.39^{b}	0.94^{a}	0.55^{b}	0.48^{b}	0.42^{b}	0.05
Apparent phosphorus retention (%)	31.08	21.24	28.12	19.83	36.64	3.51

^{abc}Means in a row with different superscripts are significantly different from each other (P<0.05) [1]Each value represents mean of 5 replicates (5 birds/replicate), PC: Positive control, NC: Negative control. [2]Standard error of mean, DM: Dietary dry matter content, DMI: dry matter Intake

In experiment 1, ileal P output accounted for 18% to 32% of P intake in diets without phytase and 8% to 10% in diets with phytase. In experiment 2, ileal output was 9% to 11% of the P intake without phytase and 6% to 7% in diets with phytase. Excreta P output accounted for between 28% and 50% P intake for the unsupplemented diets and 20% to 40% with supplemental phytase. Phytase supplementation and its interaction with P was responsible for the significant reduction of ileal and excreta P and increased apparent P digestibility, but the level of P resulted in an increase in apparent P retention in experiment 1, and in experiment 2 phytase supplementation significantly increased apparent P retention. As expected, the addition of phytase decreased P excretion. This result agrees with other authors that reported the efficacy of microbial phytase in hydrolyzing phytate P, improving its utilization by poultry and swine, and consequently leading to a reduction in P excretion [22,9].

The positive effect of phytase supplementation was evidenced in the TPD data. Phytase supplementation increased true ileal P digestibility of CM and RH with estimated TIPD values of 95.42% and 92.94%, respectively. Liu et al. [10] reported estimated TIPD for soybean meal in the range of 46 to 71. These reported findings were based on the varying calcium to phosphorus ratios used in their study. Phytase supplementation resulted in 13.27% and 17.94% increases in TIPD for CM and RH, respectively.

Simultaneously, supplementation of CM and RH with phytase increased (TP), estimated values of 92.75% and 91.83%, respectively. This translated to 12.29% and 13.61% increases. Akinmusire and Adeola [9] reported improvement in TPD of canola and soybean meal with the addition of phytase to the diets of growing pigs. Rutherfurd et al. [23] reported a 10% to 12% increase in TPD at the terminal ileum with phytase supplementation of low P diets containing soybean meal, wheat bran, and rapeseed meal in broiler chickens with a corresponding 10.5% increase in phytate degradation at the terminal ileum. Phytase supplementation had also been reported to improve Ca and P retention in broiler chickens [24] and digestible P in pigs, with a reduction in excreted P by 21.5% [20]. The results obtained in these studies on improved ileal digestible P and a corresponding P retention in both experiments agreed with these earlier findings.

A comparison of the TIPD and TPR estimates obtained at the sites of sampling for diets with and without phytase indicated significant variation, which attests to the influence of hindgut microflora on phosphorus digestibility and retention. Ileal digestible P is not influenced by hindgut microflora, because it is the portion of dietary total P that is not found in the faeces, whereas P retention is the proportion of dietary total P that is retained in the body, which could be affected by hindgut microflora [16]. Percentage increases at both sites of sampling were observed to be highest in the RH diet compared with values obtained for CM. The lower TPD in CM in relation to RH could be attributed to the antinutritional factors in CM, which is reported, apart from phytic acid, to contain antinutrients such as gossypol, which are known to reduce nutrient digestibility in non-ruminant animals [25]. It could also be attributed to the presence of adequate substrate for microbial phytase action [25]. Dersjant-Li et al. [25] concluded that the efficacy of phytase depends on the concentration of phytate in feed

ingredients, which was categorized under dietary-related factors. The difference in processing methods used to produce CM and RH probably resulted in varying concentrations of phytate in their by-products. Hence better substrate-to-enzyme concentration might have triggered the observed percentage point increase in true ileal P digestibility and TPR for RH.

The results from the two experiments show that irrespective of the assayed feedstuff, there was a similarity in the effect of phytase on P digestibility. Phytase had no effect on P retention in experiment 1 but had a significant effect in experiment 2 (Table 3). Similar effects on ileal P digestibility and retention were reported by Leytem et al. [26], who recorded an increase in the hydrolysis of phytate P and a threefold increase in P retention in a study with corn, wheat, barley, and oats in broiler chickens as a result of the addition of 1000 phytase units to the diets. Phytase supplementation has also been reported to improve digestible P in pigs with a reduction in excreta P by 21.5% [27]. These reports support the results in this study of improved ileal digestible P and a corresponding P retention in both experiments.

Estimates of EPL that were observed for broiler chickens in the two studies were not statistically different from zero. Phytase supplementation of CM and RH reduced ileal endogenous phosphorus loss and the total tract endogenous flow of P. Estimated ileal endogenous P loss for birds fed diets with and without phytase (g/kg DMI) were 0.171 and 0.211 for experiment 1, and 0.014 and 0.384 for experiment 2. This implied that supplementation of the test ingredients with phytase at 1000 units per kg of diets reduced estimated ileal endogenous phosphorus losses. Simultaneously, at the total tract section, estimated total tract endogenous P loss for birds fed diets with and without phytase were 0.581 and 0.620 g/kg DMI, and 0.338 and 0.766 g/kg DMI for experiment 1 and experiment 2, respectively. Negative estimate values were not observed in endogenous P flows at both sites of sampling for the RH diet, whereas negative values were seen in the CM diet. However, reported negative values reflect the anomaly associated with regression method, and show the drawback inherent in it. Research findings have documented negative endogenous P loss at ileal and total tract sections for pigs [9] and poultry [7,11] with more reports being documented for pigs than for broiler chickens.

In experiment 3 CM diets, Ileal phosphorus significantly ($P<0.05$) reduced with a corresponding increase in the percentage apparent phosphorus digestibility and apparent P retention with a significant reduction in excreta P with the supplementation of the combination of vit D_3 and phytase. In the RH deits, ileal P reduced with a corresponding increase in APD in D3. Supplementation of vit D3 and phytase in combination did not have any effect on APR and excreta output. Phytase and vit D_3 supplementation has been reported to improve the amount of digestible P in plant feedstuffs and consequently reduce P loss from feed ingredients [9,7] as seen from the results of these studies.

4. CONCLUSIONS

Supplementation of cottonseed meal and rice husk-based diets with phytase at 1000 units/kg of diet reduced endogenous phosphorus losses at both sites of sampling (ileal and total tract sections) in experiments 1 and 2. It also led to an increase in true ileal phosphorus digestibility and true total tract retention of phosphorus in both diets. Supplemental vitamin D_3 improved total tract phosphorus retention in cottonseed meal-based diets. The optimum inclusion level for rice husk was 456 g/kg which improved total tract phosphorus retention. However, from the results, the higher percentage point increase in true ileal digestibility and true total tract retention of phosphorus observed in phytase-supplemented rice husk than cottonseed meal strongly indicate the influence of the method of oil extraction on the concentration of phytate in the resultant meal or cake. Further study should be carried out to investigate the effect of phytase supplementation and method of oil extraction in oil-seed meals on true ileal phosphorus digestibility and retention in broiler chickens. Awareness of supplementing poultry diets with phytase should be intensified because the use of exogenous phytase would not only minimize eutrophication but decrease excessive dependence on inorganic phosphates in poultry.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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