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RESEARCH ARTICLES

Bioscience Biotechnology Research Communications

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CONTENTS

Research Design Approaches in Medical and Clinical Sciences: Assumptions, Strength, and Weakness

Bartholomew Chukwuebuka Nwogueze[1]*, Mary Isioma Ofili[2], and Elizabeth Osita Egbule[3]

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ABSTRACT

Basic research is appropriate for finding overarching principles of human behavior and biophysiological processes, whereas applied research is intended to demonstrate how these principles can be used to solve problems in a healthcare setting. Hence, this review paper examined the research design approaches in medical and clinical sciences, considering its strengths and weaknesses. Research process consists of the necessary steps or series of actions required to conduct a scientific research effectively and the desired sequencing of these steps. Research methods are classified into various types, which majorly include qualitative, quantitative, and mixed methods. Qualitative research could be phenomenological, grounded theory, ethnographic, or exploratory-descriptive in nature. Quantitative research uses numerical data to obtain knowledge about the world. Quantitative research conducted in medical and clinical sciences could involve; descriptive research, correlational research, quasi-experimental research, or experimental research in nature. However, qualitative research can also be helpful in examining subjects about which there is little information and in understanding subjective data. Mixed research methods combine quantitative and qualitative research methods in a single study. In general, the knowledge of research design can help researcher better plan the project utilizing the most appropriate methodologies and techniques.

KEY WORDS: RESEARCH DESIGN, QUANTITATIVE RESEARCH, QUALITATIVE RESEARCH, MIXED METHOD RESEARCH, MEDICAL AND CLINICAL SCIENCES.

INTRODUCTION

Research involves a systematic and controlled investigation through which data is collected, organized, analyzed, and interpreted to eliminate difficulties and improve conditions (Kerlinger, 1986). Research is a careful, systematic, organized pertinent study and investigation carried out in some field of knowledge to gather data or information to establish facts or principles. Leedy & Ormrod (2014) maintained that research is conducted for the purpose of description, exploring, explaining, and predicting. The fundamental principles that guide scientific research are empiricism, replicability, objectivity, systematic observation, reliability, accuracy, predictability, ethics, and generalization. Research is primarily committed to establishing systematic, reliable, and valid knowledge. As such, the purpose of conducting research includes; scientific study, generating new knowledge and/or finding truth, improving understanding, formulating/reviewing theories, clarifying fact, refining existing research method, decision making process for effective planning, program, and implementation, practical contribution and solving problem (Denzin & Lincoln, 2005).

A research design guides the planning and implementation of a conceived study to adequately answer generated questions or test-formulated hypotheses raised for investigation. Knowledge of the different research designs will select the design(s) appropriate for his study. Each design offers a unique approach or plan to answer the research question. Research approaches are plans and research procedures that span the steps from broad assumptions to detailed methods of data collection, analysis, and interpretation. This plan involves several decisions that involve an approach that should be used to study a topic (Cohen et al., 2011). The philosophical presumptions of the researcher, the inquiry processes (referred to as research designs), and the particular research techniques for gathering, analyzing, and interpreting data should be taken into caccount when making this choice. The type of investigation being conducted, the topic or problem of the research, the target audience for the

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study, and the individual experience of the researcher all play a significant role in the choice of research method to be used (Streefkerk, 2022).

Research conducted in medical and clinical sciences varies considerably. Probably, most researchers are familiar with clinical research, basic (laboratory) research, healthcare (services) research, educational research, and health systems (policy) research. The research approach involves a systematic process that clearly defines the objective, managing the generated data, and communicating the research findings within established framework (research design) and in accordance with existing guidelines. Frameworks and guidelines provide researchers with an indication of what to include in the research, how to carry out the research, and what types of inferences are probable based on the collected data (Pandey & Pandey, 2015). Research in the medical and clinical sciences to solve everyday practical health-related problems. When organizing such a study, researchers must consider the presumptions they will apply, the research design, and the particular technique or procedures that will be adopted to carry out the strategy to achieve a desirable outcome (Lincoln et al., 2011).

Medical and Clinical sciences utilize either qualitative, quantitative or mixed methods; however, the researcher is at liberty to choose whatever kind of study to undertake from these three options. Researches involving the use of words (qualitative research) as opposed to figures (quantitative research) or closed-ended questions (quantitative hypothesis) compared to open-ended questions (qualitative interview questions) are common ways to distinguish between the types of research design to be adopted in a study. Both quantitative and qualitative research methods are intended to answer a particular kind of research issue, and examine and investigate the many claims of knowledge (Berg & Howard, 2012). The qualitative approach helps the researcher to investigate and acquire a deeper understanding the complexity of a phenomenon (subjective measurement), whereas the quantitative method offers an objective measurement of reality (Mertens, 2003).

Researchers therefore decides whether to use a qualitative approach, quantitative approach, or a mixed method approach when designing a medical or clinical research project. The latter strategy is based on the combination of quantitative and qualitative research methods (Kothari, 2014). However, the study problem or subject being researched, the audience for whom the researcher writes, and the researcher's own experiences all have an additional impact in determining the research approach of interest to be adopted in solving health related-problems in medical and clinical sciences.

Research Design: A research design is the framework or guide used for the planning, implementation, and analysis of a study. Research design refers to the overall strategy or plan for conducting a research study. A research design implies the procedural plan or framework adopted by a researcher in answering the generated questions validly, objectively, accurately and economically in a given research (Kumar, 2005). Research design involving qualitative, quantitative, and mixed method approaches constitutes the forms of inquiry that offer particular guidance for the procedures within a study design (Pathak, 2011). Research design is essential, considering that it sets the pathway for the study as a whole and ensures that the research in medical and clinical sciences are conducted meticulously and precisely. In addition, it describes the core aim and research objectives being adopted in a given study, as well as the research protocols and techniques employed in gathering and processing statisitcal data. In other words, a research design is the plan or blue print for testing formulated hypothesis or answering research questions generated in medical and clinical sciences research.

Research design equally outlines the fundamental techniques used by researchers to produce reliable and understandable findings. It therefore implies that some of the most significant methodological choices made by researchers are incorporated into the research design (Kerlinger, 1986). When conducting research in medical and clinical sciences, we must focus on a particular design that provides a framework for the study intervention and treatment, as this provides the research with a sense of the statistical methods and logistical arrangements needed in collecting, coding, presenting, analyzing, and interpreting the statistical data as it relates to health-related issues under study. Thus, before starting a research endeavor in medical and clinical sciences, it is critical and pertinent to comprehend and adopt a suitable research design for such study.

Research Design Used in Medical and Clinical Sciences: In the medical and clinical sciences, research design is used to investigate the causes, prevention, and treatment of diseases. Researchers in this field employ several methodologies, including case-control, cohort, and randomized controlled trials, to examine various aspects of health and health-related problems (Buiting et al., 2011). Types of research designs used in medical and clinical sciences falls into three broad categories, namely: quantitative research, qualitative research, and mixed methods. This is described as follows;

Quantitative Research: In formal, objective, systematic processes like quantitative research, numerical data are employed to gather knowledge about the outside world. Quantitative research design is aimed at discovering how many people think, act, or feel in a specific way. Quantitative research emerged around 1250 A.D. and was driven by investigators with the need to quantify data. Since then, the use of quantitative research as a means of generating new knowledge and understanding has taken center stage in western culture. A numerical or statistical approach to research design is what makes up a quantitative research method. According to Leedy & Ormrod (2014), because quantitative research expands on preexisting theories, it is specific in its surveying and experimentation approach. The methodology of a quantitative research maintains the assumption of an empiricist paradigm (Creswell, 2014). Such research, is probably independent of the researcher. Consequently, data are employed to measure reality

objectively. This implies that meaning is produced by quantitative research via objectivity found in the gathered data.

The method of assessing objective theories by looking at the relationship between variables is called quantitative research. In turn, these variables can be monitored, usually with devices, allowing for the statistical analysis of numbered data. The final written report follows a predetermined format that includes an introduction, methods, analyzes, review of findings, theory, and literatures, and discussion. Similar to qualitative researchers, people conducting this type of research have presumptions about the deductive testing of ideas, the inclusion of bias safeguards, the control of alternative explanations, and the ability to generalize and replicate the results. The purpose of quantitative research is to examine the relationship between variables, such as dependent, independent, and extraneous variables (Creswell, 2014). Data sources for quantitative research includes; ordinal or cardinal data from surveys, financial reporting, census reports, test scores, demographics, and/or observations. The analytic techniques used include; Descriptive statistics, regression, regression discontinuity and hierarchical linear modeling.

Quantitative research process involves: large sample sizes, concentrating on the quantity of responses. Methods of measurement commonly used in quantitative research include scales (nominal, ordinal, interval, and ratio), questionnaires (open and closed ended), and physiological measures (control, case, intervention and treatment etc). The data collected in quantitative research are numbers that are analyzed with statistical techniques to determine the results. In quantitative research design, closed-ended questions are typically preferred. Respondents typically will not be able to provide long, open-ended answers when given a predetermined selection of options. The goal of quantitative research is to apply the results outside the context in which it was conducted. The results of in-depth studies in medical and clinical sciences may be extrapolated to other people and environments. Quantitative research methods applicable in medical and clinical sciences include; experimental research, quasi experimental research, survey research, descriptive research, comparative research and correlational research (Lincoln et al., 2011).

Experimental research: Experimental research involves manipulation of variables after the research subjects have been divided into treatment groups. Experimental designs typically use random assignment, manipulation of an independent variable(s), and strict controls. Experimental research is a method of collecting information and data on a subject through observation in controlled settings. This is primarily concerned with the type of cause and effect study. It uses two sets of variables. Experimental research seeks to determine whether a specific treatment influences an outcome. A researcher uses the first set as a reference point to calculate the differences in the second set. In experimental research, the researcher identified the dependent and independent variables and sought to determine the effect of changes in the independent variables on the dependent variables. This is purely quantitative in nature and deals with future events. Experiments include true experiments, with random assignment of subjects to treatment conditions, and quasi experiments that use non-randomized assignments (Keppel, 1991).

The experimental design provides increased confidence in cause-and-effect relationships. Random assignment means that each subject had the same chance to be assigned to the control or experimental group. The use of random assignment of subjects attempts to eliminate systematic bias. Random assignment is different from random sampling. Random sampling means that each subject had an equal chance of being selected from a larger group to participate in the study. However, it is the random assignment to different conditions that distinguishes a true experimental design. When studying the direct causal or anticipated relationship between variables, real experiments must include randomization, a control group, and variable manipulation. When any of these conditions is not met, the design is no longer considered a legitimate experiment and is labelled as quasi experimental (Keppel & Wickens, 2003).

Studies in clinical and medical sciences adopts experimental research designs, and this can be animal experiment studies (Anachuna et al., 2018; Nwogueze et al., 2020; Enebeli et al., 2022; Nwogueze et al., 2023) and human or clinical experimental studies (Ofili & Ncama, 2014; Ofili et al., 2015; Nwogueze et al., 2024a; Ofili et al., 2024) in nature. The simplest of all experimental designs in medical and clinical sciences is the post-test-only control group. Other common true experimental designs include; pretest-posttest control group design, Soloman four group design, and cross-over design.

Quasi-experimental studies: The quasi-experimental research design is similar to the experimental research design, but it lacks one or more of the features of a true experiment. The effectiveness of medical and clinical interventions in foreseeing and regulating the outcomes sought for patients and families is determined through quasi-experimental research design. Quasi-experimental, like true experimental designs, examines cause-and-effect relationships between or among independent and dependent variables. However, one of the characteristics of true experimental design is missing, typically the random assignment of subjects to groups. Although, quasi-experimental designs are useful in testing the effectiveness of an intervention and thus can be considered closer to natural settings, these research designs are exposed to a greater number of threats of internal and external validity, which may decrease confidence and generalization of study findings (Fowler, 2009). The most common used quasi-experimental designs used in medical and clinical sciences are: nonequivalent group, pretest-posttest group design, control-group interrupted time series design, single-group interrupted time series design, and counter balanced design.

Survey Research: Survey approach involves research that collects information from a sample of people based on their response to questions. This kind of study permits usage of numerous techniques for participant recruitment,

instrumentation, and data collection. The methods used in survey research can be either quantitative (e.g. questionnaires having numerically rated items) or qualitative (such as using open-ended questions) or a combination of both (i.e., mixed methods). Surveys are widely used in research in social and psychological disciplines because they are frequently used to describe and examine human behavior, however, it can be used in research conducted in medical and clinical science discipline. By examining a sample of a population, survey research offers a quantitative or numerical account of the attitudes, trends, or opinions of that population. It comprises cross-sectional and longitudinal research with the goal of extrapolating the findings from a sample to the entire population, using questionnaires or structured interviews for data collection (Fowler, 2009).

Longitudinal research: this research examines data from across time. In a longitudinal study, participants are tracked over time as risk factors, health outcomes, or both are continuously or repeatedly monitored. In other words, the variables are measured repeatedly over different periods of time. These investigations come in a wide range of sizes and complexity. At one extreme, a sizable population may be researched for years. Most longitudinal studies look at the links between initial disease morbidity or death and exposure to known or suspected disease causes. The simplest design identifies a sample or cohort of subjects exposed to a risk factor and a sample of controls who were not exposed to the risk factor. The incidence of disease in each of the two groups is then assessed after prospective follow-up with each group. Estimating attributable and relative risks involves comparing incidence rates. Confounding variables can be taken into account in two ways: by matching the controls to the exposed individuals so that they have a comparable pattern of exposure to the confounder, or by determining the exposure to the confounder in each group and correcting for any variation in the statistical analysis. Case studies, historical studies, and genetic research are a few examples of this genre (Fowler, 2009).

Cross-sectional research: The gathering of pertinent information (data) at a certain point in time is what distinguishes cross-sectional studies from other types of research. Since all data are obtained and generally refer to the time at or around the time of the data collection, there is no time dimension involved in cross-sectional studies. Although, it is frequently said that data for cross-sectional studies is gathered at a specific point in time, the term "a point in time" is typically not defined or described. The study question may have an impact on the temporal dimension. Each component of the study, including the selection of study participants, the collection of data, and the definition of the conditions or qualities examined, must have a clear understanding of the temporal dimension (Fowler, 2009).

Descriptive research: Descriptive research explores new areas of research and describes situations as they exist in the world. A phenomenon or circumstance is described using this kind of study design. It involves gathering information using questionnaires, interviews, surveys, and observations. The researcher observes, describes, and documents various aspects of a phenomenon. There is no manipulation of variables or search for cause and effect related to the phenomenon. Descriptive designs describe what actually exists, determine the frequency with which it occurs, and categorize the information. Descriptive research can be useful in medical and clinical sciences to identify patterns, trends, and relationships of a given data (Keppel & Wickens, 2003).

Comparative research: Comparative research can also be called ex post facto or causal-comparative studies. Variations in the variables that naturally exist between two or more cases, subjects, or study units are described by comparative studies. Hence, when using a comparative design, researchers typically make assumptions about how two or more units' variables differ from one another. The main difference between this approach and the quasi-experimental design is the lack of researcher control of the variables (Creswell, 2009).

Correlational research: The purpose of the correlational study design is to determine whether two or more variables are related. Using such a research approach makes it easier to build and improve explanatory knowledge. It looks at relationships. Correlational designs, when compared to direct cause-effect correlations, include the systematic exploration of the nature of relationships, or associations between and among variables. Determining the direction and degree of association between variables is the goal of correlational research. Correlations analyze the direction, degree, magnitude, and strength of relationships or associations. The results of correlational studies provide the means for generating hypotheses to be tested in quasi-experimental and experimental studies (Bogdan & Biklen, 1992). Three of the most common correlational designs include; descriptive, predictive, and model testing correlational design.

Descriptive correlational designs: This types of descriptive correlation studies describes the variables and the relationships that occur naturally between and among them.

Designs for predictive correlation: The variance of one or more variables is predicted through predictive correlational research based on the variance of another variable (s). The study variables, like the experimental designs, are classified as independent (predictor) and dependent (outcome). These variables, however, are not managed and occur spontaneously.

Correlational design model testing: Correlational investigations are used to examine or pilot test potential relationships for a model or theory. The study variables, like experimental designs, are classed as independent (predictor) and dependent (outcome). The factors, however, are not adjusted and occur spontaneously.

Assumptions of quantitative research

- The following are the perceived key assumptions underlying the quantitative research approach;

- Quantitative research is concerned with questions about: how much? How many? How often? To what extent?
- Reality is objective, "out there" and independent of the researcher; therefore, reality is something that can be studied objectively;
- Research is based primarily on deductive forms of logic, and theories and hypotheses are tested in cause-effect order;
- The researcher should remain distant and independent of what is being researched;
- The goal is to develop generalization that contribute to theory that allows the researcher to predict, explain, and understand a phenomenon.
- Data analysis is mainly statistical, and the result of research is a number, or a series of numbers, presented in tables, graphs or other forms of statistics.

Strength and Weakness of Quantitative Research: The quantitative research approach has the following significant strengths. The first is that it can be administered and evaluated quickly. No time is needed in the organization before administering the survey, and the responses can be tabulated in a short period of time (Carr, 2014). Second, numerical data gathered using this approach promote effective comparisons between groups or variables, as well allows for examination of the extent of acceptance or rejection between respondents. Bryman, (2001) argued that the quantitative research approach is research that places emphasis on numbers and figures in data collection and analysis. Imperatively, the quantitative research approach can be seen as being scientific in nature. The use of statistical data for the research descriptions and analysis reduces the time and effort which the researcher would have invested in describing his result.

Third, the use of scientific methods for data collection and analysis makes generalization possible with this type of approach. The interaction made with one group can be generalized. Fourth, replicability is another benefit derivable from the use of this research approach. Since the research approach basically relies on hypotheses testing, the researcher does not need to do intelligent guesswork, he should follow clear guidelines and objectives (Lichtman, 2013). Finally, the advantage of legitimate quantitative data, that is, data which are collected rigorously, using the appropriate method and analyzed critically, is in their reliability, and therefore, can be repeated at any other time or place and still get the same results.

Quantitative research has the following weakness: Many crucial characteristics of individuals and societies are not meaningfully reduced to numbers or adequately understood without making reference to the local context in which people live. The detachment of the researcher from the participants means that he is an 'observer' or 'looking in' and can be considered a weakness within the quantitative research approach. With this type of researcher/participant relationship, it will be extremely difficult to get the in-depth study of the phenomena within its natural settings. In quantitative research, a large sample size is required; however, lack of resources often hinders effectiveness of large-scale research. Another weakness of quantitative data with regard to disaster survey, there is difficulty of in-depth description of the experience of disaster of an affected population.

Qualitative Research: A systematic subjective approach called qualitative research is used to describe and give meaning to circumstances and events in the real world. The basis of qualitative approaches is not a forecast between two variables. Rather, open exploration of a particular topic is done using qualitative methodologies. These techniques are especially helpful for examining subjects that are poorly understood and for comprehending subjective data, such as people's experiences. In qualitative research, the research process is inductive, rather than deductive, and begins with broad exploratory aims that provide a focus for study without preempting which aspects of the experience may be deemed important or relevant. When there is a knowledge gap or when little is known about a specific occurrence, experience, or notion, researchers utilize qualitative research designs. The goal of qualitative research is to understand or interpret the meanings people give to their experiences by observing people in their natural environments (Carr, 2014).

A method of investigating and understanding the meaning that individuals or groups assign to a social or human issue is called qualitative research. Emerging questions and processes, data acquired in the participant's context, inductive data analysis leading from specifics to broad themes, and the conclusions drawn by the researcher of what the data means are all part of the research process. The final written report is organized in a customizable way. In qualitative research, information is collected in the form of words that are then meaning-analyzed after being gathered through focus groups, observations, and interviews. Qualitative research findings are unique, dynamic, focus on understanding, and facilitate theory development. The purpose of qualitative research is to explore the meaning of people's experiences, the meaning of the culture of the people, and how people view a particular issue or case (Yauch & Steudel, 2010).

Qualitative research is used to explore health-related or illness-related experiences or groups where little is known, or where current understanding seems inadequate. It is also used to get fresh perspectives on topics, populations, events, or ideas that have already been studied. Although it can be utilized concurrently or sequentially, it frequently comes before quantitative work. The most distinctive characteristics of qualitative research are that the researcher is also considered an instrument of data collection and the resulting data are mainly words or narrative descriptions rather than numbers (Carr, 2014). Data sources for qualitative research includes; normative data from interviews, documents, focus groups, and/or observations. The analytic techniques used include; thematic analysis, content analysis, and analysis of frequency. Types of qualitative research are discussed as follows;

Phenomenological study: Phenomenology uses an inductive, comprehensive approach to explain an experience

from the perspective of the person experiencing it, such as the lived experience of losing a child. The purpose of phenomenological research is to describe specific phenomena of interest as they are lived and experienced by individuals. This type of inquiry is derived from philosophy and psychology and involves the researcher summarizing participants' accounts of their lived experiences with a phenomenon. The focus of phenomenological studies is on understanding what an experience means within the context of people's lives.

This is referred to as capturing the lived experience. For a phenomenological study, the researcher purposefully chooses individuals or groups that have encountered the phenomena as their sample. The focus of the study determines the inclusion and exclusion criteria. The scope of the study, the nature of the subject, and the number of interviews conducted with each participant all play a role in estimating the size of the participant pool. The substance of the experiences for multiple people who have witnessed the event is summarized in this summary (Creswell, 2003). This design usually involves interviewing people and has solid conceptual foundations. Example: The experience of being with a seriously ill child.

Grounded theory research: Grounded theory research is an inductive research technique used to formulate, test and refine a theory on a particular phenomenon. A theory is developed based on the examination of data (rather than applying a predetermined theory). Researchers use a grounded theory design when they are interested in phenomena involving the social processes underlying human experiences and behavior. The aim of a grounded theory approach is the generation of theory that comes from or is 'grounded' in the data. The primary characteristics of grounded-theory designs are theoretical sampling and the constant comparison of data with emerging categories. This process involves using multiple stages of data collection and the refinement and interrelationship of information categories (Creswell, 2003).

Data collection and analysis occur simultaneously, and each piece of new data is constantly compared and contrasted with previously identified concepts. Sample sizes tend to be larger in grounded-theory designs, when compared to other qualitative designs, because of the need for theoretical sampling. Theoretical sampling means that the selection of participants is directed by the emerging analysis. In other words, the researcher begins with a focused sample, but as different concepts emerge, the researcher sought out additional participants based on a better understanding of these concepts. To obtain the whole spectrum of experiences or complete knowledge, this method frequently involves looking for outliers and bad situations. Theoretical sampling continues until the researcher is satisfied that the theory synthesized from the data and concepts is reflective of the social process under study. Example: Examination of the relationship between self-history and anorexia nervosa eating disorders using ground theory method.

Ethnographic research: Ethnography is the research method that comes to mind when considering the study of the shared pattern of language, behaviour, and action of a culture group in a natural setting over a prolonged period. It involves research intended to provide descriptions of systems, processes, or phenomena within their specific context. It was developed by the discipline of anthropology and sociology to investigate cultures through an in-depth study of the members of the culture. With the primary goal of advancing understanding and communication, the researcher's role in ethnography is to describe the special and distinctive practices or codes of conduct of the subculture or culture. Ethnography equally involves experiencing, most often by participant observation, enquiring, through interviews and oral histories, and examining, the study of cultural documents and artifacts.

Most of the time, ethnography is equated with the extended immersion of the researcher in the culture, group, or community under study. This is often referred to as fieldwork, and the extensive notes taken by the researcher are referred to as field notes. It is crucial to take into account cultural variations in health practices, while treating patients and their families. Researchers use ethnography as a research design when seeking a deeper understanding or description of a specific culture, group, or community (Dagn & Tebeje, 2021). Data collection often involves observations and interviews. Example: Investigation of barriers to the effectiveness of opioids in the management of cancer pain in Delta State.

Case-study research: An in-depth study of a particular case, which can be descriptive, explanatory, or exploratory. A case study is described as "an intensive study about a person, a group of people, or a unit, with the aim of generalizing over several units." Case-control studies involve a description of cases with and without a pre-existing condition or exposure. A rigorous and systematic assessment of a single person, group, community, or other unit in which the researcher looks at in-depth data related to various variables has also been referred to as a case study. When it is important to gain a thorough understanding of an issue, event, or phenomenon of interest in its authentic real-life setting, the case study approach is especially helpful to use. It discusses strange occurrences.

Depending on the content, it may be qualitative or quantitative in character. The cases, subjects, or units of study can be an individual, a family, or a group. Case-control studies are more feasible than experiments in cases in which the outcome is rare or takes years to develop (Bryman, 2008). This design is also known as a case report or case study. Example; Assessment of adolescent sexuality in females of childbearing age using a qualitative case study approach or Assessment Knowledge, attitude & adherence of among Type II Diabetes Mellitus patients to a given dietary regimen using a qualitative approach (Ofili et al., 2023).

Narrative inquiry: Narrative inquiry is a broadly determined and interpreted research design that involves individual narrative accounts and the interpretation of their meaning. Narrative accounts can be obtained from a number of groups including patients, family, and caregivers.

In narrative inquiry, the researcher studies the lives and experiences of individuals or groups by asking them to talk about or recount their experiences. The researcher then retells or restores the stories that emerge after analysis of the narratives within and between individuals. The main goal of narrative inquiry is to listen and challenge preconceived notions.

Children, for instance, are frequently understood through an adult proxy, primarily their parents. When children are asked to describe their own experiences, their narrative reports are often very different. The same is true for other people in the room and patients as well. The number of participants varies and depends on the general focus and scope of the study and the amount of information gained from each narrative account. To create a meta-narrative, or overarching story, the researcher first analyzes each participant's narrative individually, then compares them with those of other participants (Bryman, 2008; Dagn & Tebeje, 2021). Example: Evaluation of the life experiences of the Alzheimer's disease trends from childhood to adulthood.

Exploratory research: Exploratory research is conducted to address an issue or problem in need of a solution or understanding. Explanatory research focuses on why questions. Answering the 'why' questions involves developing causal explanations. Causal explanations argue that phenomenon Y is affected by factor X. Qualitative researchers' use this methodology to investigate a problem area using a variety of qualitative methodology with the goal of articulating the issue of interest and fostering knowledge. The way in which researchers develop research designs is fundamentally affected by whether the research question is descriptive or explanatory. It affects the information collected (Bogdan & Biklen, 1992).

Philosophical research: Philosophical research is entirely qualitative in nature. The researcher concentrates on how other people see the research subject matter. It is a method of research that aims to critically evaluate a philosophy or school of thought in order to gather new knowledge that can be applied to create new concepts, theories, or benchmarks. It involves research into the evolution of philosophical thought in general as well as philosophical history and various philosophers (Bogdan & Biklen, 1992; Johnson & Christensen, 2012).

Historical research: Historical research aims to provide descriptions of systems, processes, or phenomena within their specific context. To understand how previous events or ideas affected the events and ideas of the present, historians collect and analyze data on historical events or ideas. In order to understand how particular events affected the ones that followed, it investigates potential causes for those events. Historical research can give a scholar adequate knowledge about potential future events in addition to aiding in the identification of linkages between past and present events (Johnson & Christensen, 2012).

The primary sources of information used in historical research include documents from the time period studied, including historical records, books, photographs, letters, and other documentary evidence. Simply said, those sources were written by those who took part in or were present during the event.

Research can also benefit from secondary source materials, such as books and articles that were written after the events. However, secondary sources of information, which usually contain content that the writers developed, utilizing a range of sources, should be used with caution because they can be more slanted. It is important to note that primary sources might potentially be prejudiced and that there is no assurance of information authenticity because the research is looking for solutions in the past. The analysis of recent or distant past events is a component of historical study (Bogdan & Biklen, 1992). Example: Evaluation of breastfeeding trends among women attending the Federal Medical Center, Asaba, Delta State.

Assumptions of qualitative research: The following are the perceived key assumptions underlying the qualitative research approach;

- Qualitative research is concerned with finding the answer to questions that begin with why? how? In what way?
- A process of building a complex and holistic picture of the phenomenon of interest, conducted in a natural setting. Multiple realities exist in any given situation.
- When conducting qualitative research, the researcher collects data consisting mostly of words, pictures, observations of events, etc. These may eventually be categorized in some way and possibly quantified. The goal is to uncover and discover patterns of theories that help explain a phenomenon of interest
- The researcher interacts with those he/she studies and actively works to minimize the distance between the researcher and those being researched.
- Determination of accuracy involves verifying the information with informants or triangulation among different sources of information.
- Research is context-bound and is based on inductive forms of logic; categories of interest emerge mainly from informants (subject).
- Collect narrative data to gain insight into phenomena of interest. Data analysis includes the coding of the data and production of a verbal synthesis.

Strength and Weakness of Qualitative Research: Qualitative research has no structured procedure and is heavily dependent on the interpretation and ingenuity of the researchers who collects, interprets, and analyze the data. It is argued that it will not be possible to conduct the same research and get the same result at any other time and place. Qualitative research is not replicable as opposed to quantitative research (Lichtman, 2013). Qualitative methods that allow researchers to explore the views of homogeneous and diverse groups of people help to unpack these differing perspectives within a community.

The primary strength of the qualitative approach to cultural assessment is the ability to investigate the underlying values,

beliefs, and assumptions. Another strength of the qualitative research approach is that the investigation is broad and open-ended and allows participants to raise issues related to their opinion. Typically, a qualitative researcher does not have a finite or preconceived set of issues to explore. In testing hypotheses, quantitative researchers try to look at cause-and-effect relationships which perhaps enable them to predict and generalize their findings to a relevant larger population. This is not possible with a qualitative research approach (Johnson & Christensen, 2012).

Despite the usefulness of a qualitative research approach for conducting research, there are still some criticisms about the efficacy of the approach. The two main disadvantages of qualitative methods are that it takes a lot of time to complete and that it may leave out anything crucial. One possible difficulty is that all researchers' views are constrained and that a specific issue may go unreported. Additionally, because qualitative research is typically unrestricted, participants have a greater say on information gathered. Another drawback of qualitative approaches is that they require labor-intensive analysis procedures, such as recoding and categorization.

Lastly, competent interviewers are necessary to carry out the core data collection operations. However, replicability is another problem associated with a qualitative research approach. Critics of this approach argue that the constructivist has abandoned scientific methods and procedures of enquiry and investigation (Cohen et al., 2011). Meanwhile, nonuse of numbers by qualitative researchers makes it difficult and impossible to simplify findings and observations. Since the approach is characterized by feelings and personal reports, it is believed that the approach cannot provide reliable and consistent data compared to using quantifiable figures (Atkins & Wallac, 2012).

Mixed Method Research: Mixed method research is an approach to inquiry that combines quantitative and qualitative research methods in a single study. The mixed methods approach to research provided researchers with an alternative to believing that quantitative and qualitative research approaches are incompatible and, in turn, their associated methods 'cannot and should not be mixed' (Mertens, 2003). Both the quantitative and qualitative components of the study are addressed by the specified research goal and questions. A mixed methods research design incorporates the collection of both qualitative and quantitative data, their integration, and the use of unique designs that may incorporate theoretical frameworks and philosophical presumptions. Depending on the objective of their study, researchers may be more focused on using a quantitative or qualitative research approach. In mixed methods research, qualitative and quantitative data are frequently collected and then analyzed (Bryman, 2012).

Methodologies in mixed method research are increasingly being used to increase the depth and breadth of understanding of medical and clinical phenomena. Mixed method research blends qualitative and quantitative research tools to broaden and deepen understanding. Selecting the right research method begin with identifying the research question and the objective of the study. Creswell, (2014) suggests that mixed method research is an approach in which the researcher collects analyze and interprets quantitative and qualitative data, integrates the two approaches in various ways, and frames the study within a specific design.

Mixed methods can be used to gain greater insight into relationships or discrepancies between qualitative and quantitative data; they can give participants a voice and a chance to share their experiences throughout the research process; and they can open up new lines of inquiry that strengthen the evidence and allow for more in-depth answers to research questions. This type of study is predicated on the idea that combining qualitative and quantitative methods yields a more thorough grasp of a research problem than each method alone. The use of mixed method can promote increased academic exchange and enhance researchers' experiences by bringing diverse viewpoints to bear on the topics under investigation.

Research topics that neither quantitative nor qualitative methods alone could address are best addressed by a mixed methods strategy. However, combining different approaches in a single study can make research more difficult to perform. As diverse research teams must become familiar with alternative research paradigms and various techniques to sample selection, data collection, data analysis, and data synthesis or integration, it frequently demands additional resources (time and staff) and more research training (Lichtman, 2013). Mixed methods research comprises different types of design categories, including; explanatory, exploratory, parallel, and nested (embedded) designs.

Explanatory sequential mixed methods: This is a research design in which the researcher first performs quantitative research, evaluates the findings, and then builds on the findings to provide a more thorough explanation using qualitative research. Because the qualitative data further explains the initial quantitative data results, it is regarded as explanatory. Because the qualitative phase comes after the first quantitative phase, it is regarded as sequential. This kind of design is common in disciplines with a strong focus on numbers (the project starts with quantitative research). However, it may be difficult to identify the quantitative results to investigate further because there are different sample sizes for different stages of the investigation (Lichtman, 2013). Example: Identify levels of stress among interns working in hospital emergency room settings or assessing awareness & utilization levels of medical students towards insecticide-treated bed nets as measure for reducing malaria (Ofili & Nwogueze, 2024)

Exploratory sequential mixed methods: This is the explanatory sequential design is a reverse sequence. Under the exploratory sequential technique, the researcher starts by examining the perspectives of the participant during a qualitative research phase. After that, the data are examined and the results are utilized to construct a second quantitative phase. The development of an instrument that best fits the study sample, the selection of relevant instruments for the quantitative follow-up phase, or the specification of variables required for a quantitative follow-up study are

all possible uses for the qualitative phase. The focus on the relevant qualitative findings to employ and the selection of samples for both research stages present unique challenges to this strategy (Lichtman, 2013). Example: Identify the highest sources of workplace stress among physicians on internship working in the hospital emergency room or assessing levels of Serum Zinc and body mass index as Trajectory for hyperlipidemia and dyslipidemia among Welders following exposure to Welding Fumes & Smoking (Nwogueze et al., 2024b)

Convergent parallel mixed methods: To provide a thorough examination of a particular research topic, researchers would sometimes converge or integrate quantitative and qualitative data in a mixed methods research design. When using this strategy, the researcher usually gathers both types of data at about the same time and combines the data to evaluate the overall findings. In this design, discrepancies or inconsistent results are clarified or further investigated (Lichtman, 2013; Yauch & Steudel, 2010). Example: Identify sources of stress for nurses working in emergency room settings, personal coping strategies used, and types of programs or support systems provided by hospitals or assess pulmonary function parameters among females having Type II Diabetes (Eke et al., 2019).

Embedded (Nested) mixed methods: The fundamental principle of this design is that either quantitative or qualitative data are contained within a broader design (like an experiment), and the data sources play a supportive role in the overall design. This design also incorporates the usage of data in a convergent or sequential manner. Example: Test an online peer support program designed to reduce workplace stress for new medical house officers working in the hospital emergency room or evaluate the cognitive behaviour of health workers as it concerns sedentary lifestyle and physical activities during COVID-19 Pandemic (Nwogueze & Ofili, 2023).

Transformative mixed methods: This design incorporates both quantitative and qualitative data, with an overall perspective provided by a theoretical lens derived from social justice or power. In this kind of investigation, the data may be sequentially arranged with one building upon the other, or they may converge.

Multiphase mixed methods: In the domains of program interventions and evaluation his approach is typical. This advanced design best understands a long-term program goal by using sequential or concurrent procedures in tandem over time.

CONCLUSION

Understanding how to select the best design to answer a research question or test a hypothesis is the first step in conducting meaningful research that can be used to generate evidence that informs medical and clinical practice. The first and most crucial stage in conducting scientifically sound research, whether qualitative or quantitative, is to narrow the research topic and select the appropriate study design to address it. Both quantitative and qualitative approaches look at and examine various knowledge claims, and both are intended to respond to a particular research issue. The qualitative approach enables the researcher to investigate and gain a deeper understanding of the intricacy of a phenomenon, while the quantitative method offers an objective measurement of reality. The goal of researchers using mixed methods design is to draw from the strengths of quantitative and qualitative research approaches while minimizing its weaknesses. Becoming familiar with the research methods used in medical and clinical sciences allows a researcher to understand them more effectively. This is crucial for the study of human beings in both health and sickness, since a wide variety of research designs can be applied.

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In-Vitro Assessment of Free Radical Scavenging Potential of Selected Stem Extracts of *Cissus quadrangularis* Using Different Solvents

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ABSTRACT

The utilization of Cissus quadrangularis and other medicinal plants in traditional and modern medicine holds promise for the prevention and management of oxidative stress-related maladies. The aim of present work was to assess the free radical scavenging potential of the selected bioactive component of stem extract using different solvent (aqueous, methanol, dichloromethane, acetone and chloroform) of the plant Cissus quadrangularis. Antioxidant activity of the selected phytochemical of stem extract of the plant Cissus quadrangularis were done by Ferric reducing antioxidant power assay, DPPH antioxidant power assay and hydroxyl radical scavenging activity. The analysis of ferric reducing antioxidant power assay indicate that all tested solvent extract have good antioxidant potential as compare to ascorbic acid which was taken as reference. Acetone extract expressed maximum antioxidant potential with EC50 value 0.17 and chloroform extract expressed minimum antioxidant potential with EC50 value 0.35. The order of ferric reducing antioxidant potential among the tested solvent are as s follow: Acetone > Dichloromethane > Methanol > Aqueous > Chloroform. The results of the present study showed that all the extracts exhibited potent antioxidant activity. The analysis of DPPH antioxidant power assay, HRS antioxidant power assay and Ferric reducing antioxidant power assay indicate that all tested solvent extracts have good antioxidant potential and among the five extracts methanolic, dichloromethane, and acetone extract respectively exhibited higher potency of free radical scavenging activity.

KEY WORDS: ANTIOXIDANT, CISSUS QUADRANGULARIS, DPPH, FRAPA, HRSA, OXIDATIVE STRESS. REACTIVE OXYGEN SPECIES.

INTRODUCTION

Oxidative stress is one of the major region for the initiation and progression of cancer, mellitus, diabetes, neurodegenerative diseases, cardiovascular diseases and inflammatory diseases among other syndromes (Arika et al., 2019). The condition of oxidative stress arises due to the excessive generation of free oxygen and nitrogen species or their inefficient quenching within the cell (Bhat A.H. et al., 2015). Free radicals, a natural byproduct of cellular metabolism, are continuously generated in the human body as a consequence of oxygen utilization by the cells. This process, known as oxidative metabolism, occurs during various physiological activities, including respiration and energy production.

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The mitochondria, often referred to as the powerhouse of the cell, are particularly implicated in this process. Free radicals are highly reactive molecules with unpaired electrons, capable of damaging cellular components such as DNA, proteins, and lipids. While the body has defense mechanisms to neutralize these harmful effects, excessive free radical production or inadequate antioxidant defenses can lead to oxidative stress, contributing to various diseases and aging processes (Moriasi et al., 2020).

Free radicals play a fundamental role in any biochemical process and constitute an essential component of aerobic life and metabolism (Tiwari 2001). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) arise from normal cellular metabolism. The prevalent ROS consist of the superoxide anion, hydrogen peroxide (H2O2), peroxyl (ROO) radicals, and reactive hydroxyl (OH) radicals. Nitrogen-derived free radicals include nitric oxide and peroxynitrite anion (ONOO) (Joyce 1987). Reactive oxygen species and reactive nitrogen species are linked to numerous pathological conditions, including atherosclerosis, ischemia, tissue reperfusion injury, central nervous system damage,

gastritis, and cancer (Manoharan et al., 2005). Endogenous sources of free radicals comprise electron transfer chain reactions in the mitochondria, the xanthine oxidase pathway, and occurrences during disease states such as inflammation, ischemia, and reperfusion injury (Moriasi et al., 2020).

Figure 1: DPPH radical scavenging activity of different stem extracts of the plant Cissus quadrangularis.

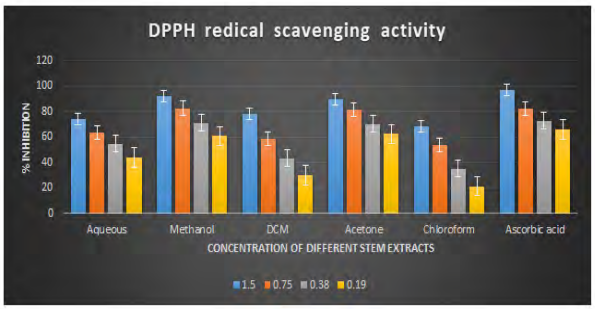


Figure 2: DPPH radical scavenging activity of different plant extracts of the plant Cissus quadrangularis.

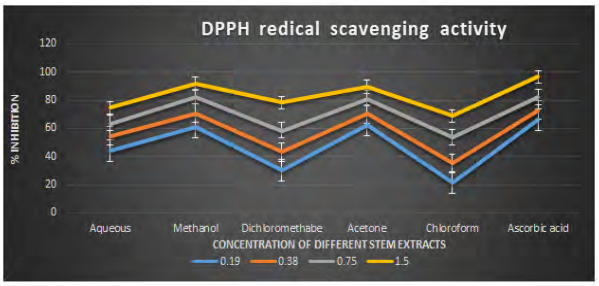
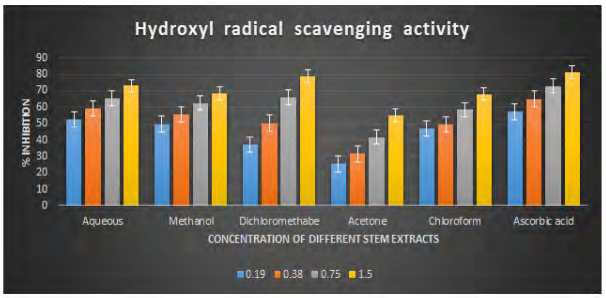


Figure 3: Hydroxyl radical scavenging activity of different plant extracts of the plant Cissus quadrangularis.



Many antioxidants are utilized to eliminate these free radicals. They exert a protective effect by neutralizing free radicals, toxic byproducts of natural cell metabolism. The human body employs various mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ or externally supplied through foods and/or supplements. These antioxidants function as free radical scavengers, preventing and repairing damages caused by ROS. Consequently, they can enhance immune defense and reduce the risk of cancer and degenerative diseases (Ganapaty et al., 2007).

Conventionally, oxidative stress is typically addressed through the utilization of various types of synthetic antioxidant compounds, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG). However, despite their widespread use, these synthetic antioxidant compounds have been linked to undesirable effects (Ndhlala et al 2010). To overcome the harmful effects of synthetic antioxidants, an available alternative is the use of medicinal plants, which offer potent, safer, more affordable, and easily accessible therapies for oxidative stress-related ailments (Goyal et al 2019).Plants are acknowledged as significant sources of novel drugs, offering key molecules of pharmacological interest. Their established applications in traditional medicines have garnered significant attention as a central focus of research (Javaid et al., 2023).

Figure 4: Hydroxyl radical scavenging activity of different plant extracts of the plant Cissus quadrangularis.

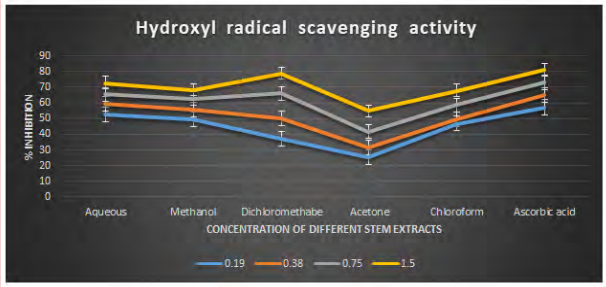


Figure5: Ferric reducing antioxidant power assay of different plant extracts of the plant Cissus quadrangularis.

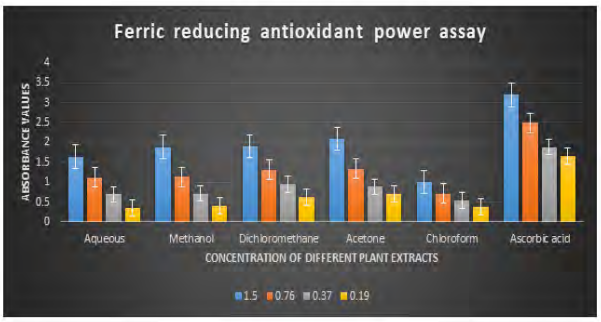
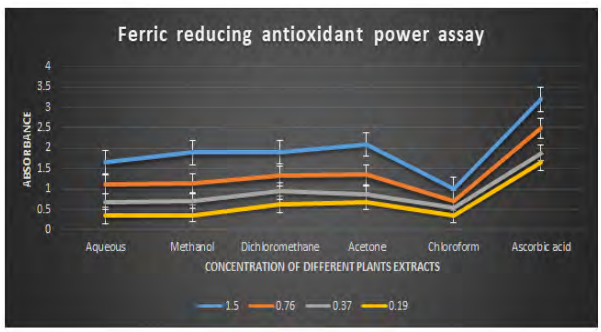


Figure 6: Ferric reducing antioxidant power assay of different plant extracts of the plant Cissus quadrangularis.



Presently, researchers are directing their focus towards phytochemicals for managing and treating various human diseases. It's noteworthy that over 50% of all modern clinical drugs originate from natural products, underscoring their pivotal role in the development of pharmaceuticals within the industry (Bardoloi et. al., 2018). Ayurveda, Unani, Siddha, and modern medicinal systems utilize numerous plants in the treatment of various diseases (Jainu et al., 2004). In the Indian subcontinent, there exists a vast array of medicinal plants that are utilized as drugs for treating numerous diseases (Ballabh et al., 2007). *Cissus quadrangularis* stands out as one of the most beneficial flora among them.

Cissus quadrangularis, a perennial herb of the grape family, features a stout, fleshy quadrangular stem with medicinal properties found throughout the tropical regions of the Earth. Also known as Cissus succulent, it is popularly referred to as horjora in Hindi and pirandai in Tamil, and belongs to the family Vitaceae. This plant is extensively utilized in India and is believed to be native to India, Sri Lanka, Malaysia, Java, and West Africa. Widely observed in tropical forest regions of Asia and Africa, *Cissus quadrangularis* is an evergreen climber that grows at a rapid rate (Ruskin et al., 2014).*Cissus quadrangularis* has been extensively studied for its phytochemical composition, pharmacological activities, and toxicological evaluation. Numerous phytochemicals, including alkaloids, tannins, lignins, suberins, phenols, flavonoids, resveratrol, piceatannol, pallidol, perthenocissin, phytosterols, and others, have been identified in the plant extract of *Cissus quadrangularis* (CQ) (Sundaran et al., 2020).

Among these, ascorbic acid, triterpenes, beta-sitosterol, ketosterol, two asymmetrical tetracyclic triterpenoids, and calcium have been recognized as the major phytochemicals of this plant (Jainu et al., 2004). *Cissus quadrangularis* exhibits a diverse range of beneficial properties, including antimicrobial, antioxidant, anti-inflammatory, anti-cancerous, and cytotoxic effects. Furthermore, it has been observed to promote bone healing, making it particularly valuable in traditional medicinal practices.

Its antimicrobial properties make it effective against various pathogens, while its antioxidant and anti-inflammatory actions contribute to overall health and wellness. Additionally, its potential anti-cancer properties offer promise in combating malignancies, and its ability to aid in bone healing underscores its importance in orthopedic medicine. These multifaceted properties highlight the potential of Cissus quadrangularis as a valuable therapeutic agent in the treatment of various ailments., Panche et al 2016, Chinthamani et al 2014, Murthi et al 2003, Kuppuramalingam et al 2018 & Rekha et al 2019, Anwar et al 2021 and Dinesh et al 2021).

MATERIALS AND METHODS

Preparation of stem extract: The fresh stems of *Cissus quadrangularis* (CQ) were harvested from Supaul district in Bihar, India (Latitude 26.5520640 and Longitude 87.0555330) (Figure 1). Authentication of the plant and stem was conducted by Prof. Rimjhim Sheel, Former Head, University Department of Botany & Dean Faculty of Science and Principal GDM College. Patliputra University, Patna. A voucher specimen has been preserved in the University Department of Botany at Patliputra University, Patna, Bihar, India for future reference. After collection, the stems were cleaned thoroughly by washing them in tap water followed by rinsing with distilled water. Subsequently, the stems were shade dried and ground into a fine powder. The powdered material was then stored in a clean, airtight container for further use. All the chemicals utilized in this study were procured from two suppliers: Bihar Scientific Company and Krishna Scientific, both located in Patna, Bihar, India.

Soxhlet extraction: The dried powder of *Cissus quadrangularis* stems (50g) was packed into the thimble of a Soxhlet apparatus. Successively, 400 ml of methanol, acetone, chloroform, dichloromethane, hexane, and aqueous solvents (referred to as MACDHA) were employed as solvents one after the other. These solvents were utilized to dissolve the active biomolecules present in the plant material. The stems remained as precipitate while the active biomolecules were extracted into the solvent. The extraction process was continued until the solvent in the thimble appeared clear, typically taking approximately 8 hours on average. Subsequently, the solvent extract was evaporated in a water bath until a dark orange residue was obtained. The percentage yield was 12%, 8%, 7%, 9%, 10% and 12% for methanol, acetone, chloroform, dichloromethane, hexane and aqueous (MACDHA) respectively. The extract were kept at -200 C till further use. All the process of Soxhlet extraction was completed in University Department of botany and Department of Botany TPS College, Patliputra University.

Estimation of free radical scavenging activity: Antioxidant activity of the selected phytochemical of stem extract of the plant *Cissus quadrangularis* were done by the following methods.

Determination of 1,1, dipheny-2-picrylhydrazyl (DPPH) Radical Scavenging Activities: The DPPH radical scavenging assay was conducted following the protocol outlined by Brand-Williams et al. with certain modifications. In brief, five different concentrations of the plant extracts under investigation (0.0625, 0.125, 0.25, 0.5, and 1 mg/ml) were prepared in methanol (analytical grade). Equivalent concentrations were also prepared for L-ascorbic acid, serving as the standard antioxidant. Subsequently, 1 ml of each plant extract was transferred into a clean test tube, to which 0.5 ml of 0.3 mM DPPH solution in methanol was added.

The mixture was then shaken and left to incubate in the dark at room temperature for 15 minutes. Blank solutions, consisting of the plant extract solutions (2.5 ml) and 1 ml of methanol, were utilized as the baseline. The negative control was composed of 2.5 ml of DPPH solution and 1 ml of methanol, while L-ascorbic acid at equivalent concentrations to the plant extracts served as the positive control.

Following the incubation period in darkness, the absorbance values were measured at 517 nm using a spectrophotometer. All experiments were conducted in triplicate. The DPPH radical scavenging activity was calculated using the equation as described by Brand-Williams et al. Where, As is absorbance of the sample and Ac is absorbance of the control. The half maximal inhibitory concentration (IC_{50}) of the extracts was computed from a plot of percentage DPPH free radical inhibition versus the extract concentration.

Determination of Hydroxyl Radical Scavenging Activity: The following steps were conducted to determine the Hydroxyl Radical Scavenging Activity of the plant extracts by the method outlined by Klein et al. (1991) with certain modifications: A solution containing 0.13% ferrous ammonium sulfate and 0.26% EDTA was prepared. 1 mL of the prepared Iron-EDTA solution was mixed with various concentrations of plant extracts. To this mixture, 0.5 mL of EDTA solution (0.018%) and 1 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%). The reaction mixture was then incubated at a temperature ranging from 80 to 90°C for 15 minutes in a water bath. After incubation, the reaction was terminated by adding 1 mL of ice-cold trichloroacetic acid (TCA) (17.5% w/v).Following TCA addition, 3 mL of Nash reagent was added to the reaction mixture. The reaction mixture was allowed to stand at room temperature for 15 minutes. The absorbance of the reaction mixture was measured spectrophotometrically at 412 nm against a reagent blank. The percentage of hydroxyl radical scavenging activity was calculated using the following formula:

$$\% \text{ Radical scavenging activity} = \frac{Ac - As}{Ac} \times 100$$

Where, As is absorbance of the sample and Ac is absorbance of the control. The half maximal inhibitory concentration (IC_{50}) of the extracts was computed from a plot of percentage DPPH free radical inhibition versus the extract concentration.

Ferric Reducing Antioxidant Power Assay: The reducing power of the extracts was assessed following the method outlined by Oyaizu et al. (1986) with slight modifications. Four different concentrations of aqueous extract (1.5 mg, 0.75 mg, 0.38 mg, and 0.19 mg) along with L-ascorbic acid at equivalent concentrations were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of 1% potassium ferricyanide. The mixtures were then incubated at 50°C for 20 minutes. Subsequently, 2 ml of 10% trichloroacetic acid was added, followed by centrifugation at 1000 rpm for 10 minutes. The supernatant (2 ml) was aspirated and mixed with 2 ml of distilled water and 1 ml of 0.1% ferric chloride. The absorbances were measured at 700 nm using a UV-Vis spectrophotometer and recorded. The concentration of each extract capable of producing an absorbance value of 0.5 was determined from the graph of absorbance at 700 nm against extract concentration. This concentration was considered as the median effective concentration (EC_{50}).

RESULT AND DISCUSSION

The stems of CQ were freshly collected from Supaul district of Bihar, India (Latitude 26.5520640 and Longitude 87.0555330). Authentication of the plant and stem was performed by Prof. Rimjhim Sheel, Former Head, University Department of Botany & Dean Faculty of Science and Principal GDM College, Patliputra University, Patna.

Reactive oxygen species (ROS) or free radicals are natural byproducts of cellular metabolic reactions. However, when they accumulate in cells, they can transform into highly toxic substances that damage essential cellular components such as DNA, RNA, proteins, and lipids (Ali S. S. et al., 2020). These accumulated damages can contribute to the development of chronic diseases including cancer, diabetes, and heart diseases (Hajam Y. A. et al., 2022). Balancing the effects of ROS is crucial, and external antioxidants play a significant role in this process. Plants, being a major part of our diet, serve as the primary source of these antioxidants (Nwozo O. S. et al., 2023).

The processing and extraction of the leaves and stems of CQ were conducted accordingly, and the extracts were obtained using various solvents (Acetone, Chloroform, Methanol, Dichloromethane, and Aqueous). These extracts were then subjected to further analysis for radical scavenging activity. The results of the scavenging activity are tabulated below.

DPPH antioxidant power assay

Comparative analysis of % inhibition by plant extract of different solvents. (Table 1)

Graphical presentation of % inhibition by plant extract of different solvents.

DPPH˙ (1,1-Diphenyl-2-picrylhydrazyl) is a stable nitrogen-centered free radical with an unpaired electron at one atom of the nitrogen bridge. The scavenging of DPPH free radicals is a widely used method for assessing antioxidant activity. This assay measures the ability of antioxidants to directly scavenge DPPH˙ radicals by monitoring changes in absorbance using a spectrophotometer at a wavelength of 517 nm (Kedare S. B. et al., 2011). The DPPH radical scavenging assay offers a quick and simple way to evaluate the antioxidant activity of various plant extracts. In this study, the Acetone, Aqueous, Chloroform, Dichloromethane, and Methanol extracts of Cissus quadrangularis stems were assessed for their ability to scavenge free radicals using DPPH˙ as the substrate.

This assay measures the hydrogen or electron donating ability of the stem extracts. The extracts of Cissus quadrangularis stems were found to reduce the stable purple color of DPPH (1,1-diphenyl-2-picrylhydrazyl) free radicals to yellow-colored 1,1-diphenyl-2-picrylhydrazine. The reduction capacity increased with increasing concentration of the extract. Analysis of the DPPH antioxidant power

assay indicated that all tested solvent extracts exhibited good antioxidant potential compared to ascorbic acid, which was used as a reference antioxidant.

The IC_{50} values for aqueous, methanol, dichloromethane, acetone and chloroform extracts are 0.28, 0.09, 0.51, 0.08, and 0.63 mg/ml respectively was compared with standard ascorbic acid (IC_{50} = 0.07 mg/ml) ($p < 0.05$; Table 1). Furthermore, it was demonstrated that the IC_{50} value for L-ascorbic acid was lower than the IC_{50} values of all the studied plant extracts. At all the tested concentration the maximum DPPH· radical scavenging activity was 91.88±0.08 % for methanol extract and minimum was 21.45±0.11 for chloroform extract among the plant extracts ($p < 0.05$; Table 1 and Fig 1).

Furthermore, it was demonstrated that the DPPH· radical scavenging activity of ascorbic acid was maximum at all the concentration against plant extracts. The decreasing order of 1,1-diphenyl-2-picrylhydrazyle radical scavenging activity of the different extract was found to be Methanol > Acetone > Dichloromethane > Aqueous > Chloroform ($p < 0.05$; Table 1 and Fig 1) with correlation coefficient 0.95, 0.96, 0.98, 0.96 and 0.96 respectively.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity (Table 2).

Graphical presentation of % inhibition by plant extract of different solvents.

Table 1. DPPH radical scavenging activity showing % inhibition of different plant extracts of the plant *Cissus quadrangularis*. Data were expressed as Mean ± Standard Deviations (SD). Values differ significantly at p<0.05

S. N.	Concentration (in mgs)	% inhibition (By plant extract of different solvents)					
		Aqueous	Methanol	Dichlorome-thane	Acetone	Chloroform	Ascorbic acid
01	1.50	74.43±0.16	91.88±0.08	78.42±0.22	89.64±0.14	68.72±0.22	96.72±0.06
02	0.75	63.82±0.20	82.59±0.08	58.67±0.16	81.32±0.12	53.90±0.12	82.33±0.04
03	0.38	50.46±0.19	70.87±0.06	43.37±0.17	70.42±0.06	35.30±0.23	72.88±0.07
04	0.19	43.98±0.11	60.77±0.11	30.14±0.19	62.39±0.22	21.45±0.11	66.06±0.09
IC_{50} (in mgs)		00.28	00.09	00.51	00.08	00.63	00.07

Table 2. Hydroxyl radical scavenging activity showing % inhibition of different plant extracts of the plant Cissus quadrangularis. Data were expressed as Mean ± Standard Deviations (SD). Values differ significantly at p<0.05.

S. N.	Concentration (in mgs)	% inhibition (By plant extract of different solvents)					
		Aqueous	Methanol	Dichlorome-thane	Acetone	Chloroform	Ascorbic acid
01	1.50	73.03±0.07	68.38±0.21	78.90±0.26	54.82±0.10	68.09±0.29	81.45±0.20
02	0.75	65.35±0.03	62.61±0.17	65.97±0.10	41.69±0.40	58.46±0.21	73.00±0.21
03	0.38	59.34±0.07	55.61±0.20	50.31±0.20	31.49±0.32	49.32±0.16	65.00±0.20
04	0.19	52.65±0.17	49.69±0.17	37.19±0.69	25.43±0.08	46.93±0.23	57.26±0.20
IC_{50} (in mgs)		00.16	00.19	00.37	01.23	00.36	00.11

The hydroxyl radical, being the most reactive oxygen-centered species, can cause significant damage to adjacent biomolecules. In this study, the hydroxyl radical scavenging assay was conducted by generating hydroxyl radicals using ascorbic acid and EDTA. The hydroxyl radicals were formed through an oxidation reaction with dimethyl sulfoxide (DMSO), resulting in the production of formaldehyde, which provided a convenient method for detecting hydroxyl radicals by treatment with Nash reagent. All the extracts of Cissus quadrangularis, when added to the reaction mixture, exhibited the ability to scavenge hydroxyl radicals in a concentration-dependent manner.

This scavenging activity could be attributed to the presence of phenolic compounds in the extracts, which possess hydrogen-donating abilities. Analysis of the hydroxyl radical antioxidant power assay revealed that all tested solvent extracts (aqueous, methanol, dichloromethane, acetone, and chloroform) displayed significant antioxidant potential compared to ascorbic acid, which served as the reference antioxidant in this study.

The IC_{50} values for aqueous, methanol, dichloromethane, acetone and chloroform extracts are 0.16, 0.19, 0.37, 1.23, and 0.36 mg/ml respectively was compared with standard

ascorbic acid (IC_{50} = 0.11 mg/ml) ($p < 0.05$; Table 3) Furthermore, it was demonstrated that the IC_{50} value for L-ascorbic acid was lower than the IC_{50} values of all the studied plant extracts. At all the tested concentration the maximum hydroxyl radical scavenging activity was 78.90±0.26 % for dichloromethane extract and minimum was 25.43±0.08 for acetone extract among the plant extracts ($p < 0.05$; Table 3 and Fig 3). The decreasing order of hydroxyl radical scavenging activity of the different extract was found to be Dichloromethane > Aqueous > Methanol > Chloroform > Acetone ($p < 0.05$; Table 3 and Fig 3) with correlation coefficient 0.96, 0.97, 0.96, 0.99, and 0.99 respectively.

Ferric reducing antioxidant power assay

Comparative analysis of absorption of different solvents (Table 3).

Table 2. Hydroxyl radical scavenging activity showing % inhibition of different plant extracts of the plant Cissus quadrangularis. Data were expressed as Mean ± Standard Deviations (SD). Values differ significantly at p<0.05.

S. N.	Concentration different solvents	Absorbance					
		Aqueous	Methanol	Dichlorome-thane	Acetone	Chloroform	Ascorbic acid
01	1.50	1.638±0.001	1.880±0.001	1.901±0.001	2.089±0.002	1.003±0.003	3.192±0.002
02	0.75	1.112±0.001	1.137±0.001	1.306±0.004	1.332±0.002	0.716±0.005	2.455±0.002
03	0.39	0.693±0.001	0.712±0.001	0.9560.002	0.884±0.003	0.540±0.001	1.885±0.001
04	0.19	0.342±0.001	0.394±0.001	0.62±0.001	0.692±0±0	0.383±0.003	1.664±0.004
EC_{50} (in mgs)		00.23	00.23	00.18	00.17	00.35	00.05

Graphical presentation of absorbance value of Ferric reducing antioxidant power assay by plant extract of different solvents.

This method is based on the ability of the analyte to reduce ferric ions (Fe3+) to ferrous ions (Fe2+) (Gulcin I., 2010 & MacDonald-Wicks L. K. et al., 2006). Therefore, the formation of Fe2+ can be assessed by measuring the absorbance capacity at 700 nm. Increases in absorbance at this wavelength indicate an increase in reducing power. The analysis of the ferric reducing antioxidant power assay revealed remarkable concentration-dependent increases in absorbance values at a wavelength of 700 nm (Table 2) compared to ascorbic acid, which was used as the reference antioxidant in this study. The half-effective concentrations (EC50) of the studied plant extracts required to produce an absorbance value of 0.5 were determined in this study.

The EC50 values for aqueous, methanol, dichloromethane, acetone and chloroform extracts are 0.23, 0.23, 0.18, 0.17, and 0.35 mg/ml respectively was compared with standard ascorbic acid (IC_{50} = 0.07 mg/ml) ($P < 0.05$; Table 2). Furthermore, it was demonstrated that the EC50 value for L-ascorbic acid was lower than the EC50 values of all the studied plant extracts. At all the tested concentration the maximum absorbance value for ferric reducing antioxidant activity was 2.089±0.002 for acetone extract and minimum was 0.342±0.001 for aqueous extract ($p < 0.05$; Table 2 and Fig 2).

Furthermore, it was demonstrated that the absorbance value for ferric reducing antioxidant activity of ascorbic acid was significantly maximum at all the concentration against plant extracts (table 2). The decreasing order of ferric reducing antioxidant activity of the different extract was found to be Acetone > Dichloromethane > Methanol > Aqueous > Chloroform ($p < 0.05$; Table 2 and Fig 2) with correlation coefficient 0.99, 0.99, 0.99, 0.98 and 0.99 respectively.

CONCLUSION

Today, there is a growing interest in the antioxidative properties of plants due to their potential use as natural additives to replace synthetic ones. The results of the present study demonstrate that all the extracts exhibited potent antioxidant activity. Analysis of the DPPH antioxidant power assay, HRS antioxidant power assay, and Ferric reducing antioxidant power assay indicated that all tested solvent extracts possessed good antioxidant potential. Among the five extracts, methanolic, dichloromethane, and acetone extracts respectively exhibited higher potency of free radical scavenging activity. These findings suggest that the stem extract of the plant Cissus quadrangularis could serve as a valuable source of natural antioxidants for promoting health benefits. Further isolation of bioactive compounds is recommended to identify the unknown compounds and establish their pharmacological properties.

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Analysis of Coagulation Profile and Possible Mechanism of Coagulation Activation in COVID-19 Patients: A Systematic Literature Review

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ABSTRACT

The ongoing COVID-19 pandemic has caused a global health crisis with serious impacts that extending beyond the acute infection stage. This comprehensive review of literature looks at the mechanism of coagulation activation during COVID-19 as well as the examination of coagulation profile in infected patients. Prolonged prothrombin time (PT), higher D-Dimer, thrombocytopenia, and altered coagulation factors activity are indicators of hypercoagulability in severe cases. Long-term COVID-19 sequelae indicated persistent difficulties, such as psychological and physical disorders, that are revealed throughout the post-recovery phase. Multiple studies have shown a correlation between the severity of the disease and coagulation issues, highlighting the importance of a thorough coagulation profile investigations in COVID-19 patients after recovery.

Mechanistically, the inflammatory response initiates a cytokine storm that activates monocytes, platelets, and endothelial cells. The viral spike protein interacts with the ACE2 receptors of endothelial cells, causing endothelial damage and activating procoagulant pathways. In addition, alterations in the renin-angiotensin system (RAS) intensify vasoconstriction, inflammation, and pro coagulation. Recognizing these intricate biochemical processes, it is essential to predict and manage chronic consequences related to coagulation factors. In patients who have recovered, early examinations of coagulation parameters may help with early medications, which could prevent thrombotic incidents.

KEY WORDS: COAGULATION FACTORS, COVID-19, D-DIMER, ENDOTHELIAL DYSFUNCTION, VENOUS THROMBOEMBOLISM,

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a global health crisis that emerged in Wuhan City, China on December 3, 2019, and is still an ongoing pandemic worldwide. According to data from the Indian Government, India is ranked second in the world, with 44,996,963 confirmed COVID-19 cases and had the third highest number of 531,928 COVID-19 deaths (Ritchie et al. 2022). Although the patients recovered from infection, after effects of COVID-19 do not end with infection resolution, as reported in studies (Del Rio, Collins and Malani, 2020), patients after 12 weeks of COVID-19 recovery experienced a wide range of mental and physical complications with specific organ dysfunctions involving heart, lung and brain, and stated that major consequences of COVID-19 increased the incidences of heart failure in young population and athletes and also decline in lung functions and neurological manifestations. Studies also support the hypothesis of severe major consequences reported earlier due to long COVID-19 sequelae by examining blood samples from 70 South African long COVID-19 patients, (Pretorius et al., (2021).

All the results showed platelet pathology and substantial fibrin amyloid microclots which can be linked to chronic symptoms that persisted even after the subsidence of acute COVID-19. Coagulopathy is an emerging hallmarks of COVID-19 induced by the hyperinflammatory response. The authors also found that elevated coagulation parameters such as prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer, and thrombocytopenia are associated with higher mortality from COVID-19. Hence, examination of these factors is important to determine the level of coagulopathy, (Wang et al 2020).

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The major issue that needs to be addressed is the emergence of mysterious clots that result in coagulation irregularities and thrombosis. An earlier detection of thrombotic events will be possible by the increased knowledge of thrombotic consequences in recovered individuals. The primary objective of this literature review is to analyze studies investigating coagulation parameters in COVID-19 patients and to provide an overview of the mechanisms underlying COVID-19-associated coagulopathy.

MATERIAL AND METHODS

Search Strategy: PubMed, Scopus, Web of Science, and Google Scholar databases were searched to identify relevant articles. Following key words were used for the search ensuring an extensive search strategy, literature search performed using COVID-19 and coagulopathy related keywords such as "COVID-19", "coagulation factors", "coagulopathy", "thrombosis", "D-Dimer", "coagulation mechanism".Inclusion and exclusion Criteria: In order to assure relevance to recent advances in the understanding of COVID-19 associated coagulopathy, only peer reviewed articles written in English between 2019 and 2023 are taken into consideration. Articles that did not meet the inclusion criteria were also excluded.

Selection of studies: Screening: The titles and abstracts if retrieved papers were examined to determine their relevance to the goal of the study.

Full-text Review: To ascertain eligibility in accordance with the inclusion and exclusion criteria, potentially relevant papers were subjected to a full-text review.

Extracting data: Relevant information about COVID-19-associated coagulopathy, thrombosis risk, population characteristics, study design, and measured coagulation parameters were gathered from a subset of selected studies.The publications that offered significant insights into COVID-19-associated coagulopathy and its clinical consequences are included and served as the foundation for our review and can help to unfold later pathological manifestations. The included papers' references were taken out and subjected to additional examination and analysis. This made it possible to find other information sources and to investigate similar studies that might advance our grasp of the subject matter.

RESULTS AND DISCUSSION

The level of coagulation parameters changes due to the COVID-19 severity (Teimury, Khameneh and Khaledi, 2022). They reported that patients with severe COVID-19 had decreased lymphocytes, reduced platelet count (thrombocytopenia), high fibrinogen and fibrin degradation products (FDP), highly increased D-dimer, higher factor VIII activity, lower factor V and VII activity, elevated PT and APTT, and low antithrombin III (AT III).

To investigate the difference between survivors and non-survivors, coagulation parameters of consecutive novel coronavirus pneumonia (NCP) cases were studied in Tongji Hospital, Wuhan China, and vigorous fluctuations in parameters were traced. They reported the D-dimer range 0.22-21.00 µg/ml and the FDP range of 4.0-150.0 µg/ml and concluded that significantly higher coagulation parameters could be associated with the development of coagulation disorders (Wang et al., 2020).

Analysis of coagulation parameters performed to determine the consumption of coagulation factors and found normal median PT, and APTT but particularly elevated D-dimer (median 450 ng/ml), and mean fibrinogen levels were also above the upper limit. Workers (Martín-Rojas et al., 2020) also observed that 11 out of 206 patients met the criteria for overt DIC (disseminated intravascular coagulopathy) with elevated D-dimer levels (median 2812 ng/ml). Prolonged PT (median 16.5 S), lower platelet count (median 98×103 µl), decreased level of protein C and antithrombin, lower level of factor II, X and XII .

A study (Cui et al., 2020) explored the incidence of venous thromboembolism (VTE) in ICU patients with severe NCP and investigated the difference between VTE and non-VTE patients. Patients with VTE had higher D-dimer, longer APTT, and lower lymphocyte counts, and these parameters were also consistent with those of older patients. Researchers (McFadyen, Stevens and Peter, 2020) reported that, patients with COVID-19 have markedly increased rates of venous thromboembolism (VTE) and pulmonary embolism. In addition, arterial thrombosis, acute myocardial injury, and microvascular thrombosis commonly complicate the condition of patients. Elevated acute-phase reactants such as C-reactive protein (CRP) and fibrinogen and abnormal coagulation parameters such as prolonged APTT, PT, D-dimer, thrombocytopenia (<100×10^9/L) are prognostic markers in COVID-19.

Whole blood samples from 24 COVID-19 patients were analyzed to evaluate parameters by Thromboelastography (TEG), an in vitro device used to assess the viscoelastic properties of native whole blood upon stimulation of hemostasis by an exogenous trigger (kaolin); they concluded that COVID-19 patients show hypercoagulability, which could develop pulmonary embolism or deep vein thrombosis of the lower limbs (Panigada et al., 2020). They also observed other parameters of hemostasis such as normal or slightly prolonged PT and APTT, greatly increased fibrinogen and D-dimer levels, and suggested that patients with COVID-19 do not have DIC; rather they support hypercoagulability together with a severe inflammatory state.

Their findings are also supported by a study (Levi et al., 2020), which reported that patients with COVID-19 coagulopathy not have many hemorrhagic complications and excessive thrombin generation, which is the characteristic feature of DIC. The clinical and laboratory features of coagulation changes in COVID-19 did not match the DIC score of the International Society on Thrombosis and Haemostasis (ISTH). Therefore, they concluded that COVID-19 coagulopathy is distinctly different from DIC. The proportion of abnormalities was higher in the severe group than in the mild group, and significant coagulopathy

was correlated with the degree of disease severity to some extent (Zou et al., 2020). This analysis inferred by studying coagulation parameters in 303 COVID-19 patients in Shanghai, China. The abnormal parameters were fibrinogen in 64.3% patients, D-dimer in 42.6% patients, prolonged prothrombin time in 18.5% patients, abnormal activated partial thromboplastin time in 21.8% patients, and elevated fibrinogen degradation products in 6.3% patients.

The study js also supported by the findings of Abd El-Lateef et al., (2022) who analyzed the differences in coagulation markers and biochemical and inflammatory markers in the severe and non-severe patients and found increased PT, INR, APTT, D-dimer, fibrinogen, C-reactive protein (CRP), factor VIII, VWF and ristocetin cofactor (RiCoF) and decrease lymphocyte count in severe patients but with not any variation in platelet counts.

However, there were significant differences between survivors and non-survivors. All the biochemical inflammatory and coagulation markers were greatly increased in non-survivors, with a decreased lymphocyte and platelet counts. They also found RiCoF was a novel predictor of COVID-19 severity. RiCoF forms complexes with VWF and induces platelet aggregation by conformational change in VWF. Another study by Al Nafea et al., (2023) also depicts coagulopathy associated with severe COVID-19 patients by assessing coagulation profile of survivors and non-survivors and concluded that non-survivors exhibited higher level of D-Dimer (36.8%), PT (31.5%) and PTT (10.5%), demonstrating a strong association between coagulopathy and disease severity.

Coagulation profile of 455 hospitalized COVID-19 patients analyzed in Addis Ababa, Ethiopia, of which 46% showed prolonged PT and variation in INR values. Prolonged PT were more frequent (51.3%) in older people (> 55 years) and males (49.8%) than in females (41%), and 22.1% of total patients had thrombocytopenia. Venous thromboembolism (VTE), arterial thrombosis, and thrombi in vessels of the lung, kidney, and other organs have been reported in critically ill patients with COVID-19 (Araya et al., 2021).

Another study by Larsen, Pasalic and Hvas, (2020) also reported that patients with COVID-19 frequently have minor thrombocytopenia; nonetheless, it is uncommon and should be considered as a sign of either existing or emerging thrombocytopenia when the platelet count is <100×109 /L. They also reviewed the mechanism behind it, which may be adhesion and activation of platelets and this is due to direct influence of virus on hemostasis. In an investigation by Bilaloglu et al., (2020) conducted on 3334 consecutive COVID-19 hospitalized patients, the authors stated that higher D-dimer levels were related to a thrombotic event and that 533 (16.0%) patients experienced such occurrences.

D-dimer level in 119 COVID-19 patients who recovered within the last 6 months were assessed by Lehmann et al., (2021), and elevated D-dimer levels were found in 15% of the patients who had severe COVID-19 that required hospitalization. Of these, when CT scan performed in 79% patients showed elevated D-dimer levels, 13% patients had thrombotic complications. Therefore, D-dimer could be a potential biomarker for post-COVID-19 conditions. Their hypothesis was also supported by another study that assessed the coagulation profile of 75 children below 18 years of age and had confirmed COVID (Di Gennaro et al., 2022).

Tests were performed after 8-12 months of recovery. The coagulation profile of children with post-COVID conditions (PCC) who had at least three or more persisting symptoms compared with the control group of children fully recovered post-SARS-COV-2. They found that the majority of children displayed a coagulation profile that was near normal or within normal range but had significantly elevated D-dimer levels in children with post-COVID conditions compared to those fully recovered from infection.

Mechanism of coagulation activation in patients with COVID-19: COVID-19 coagulopathy also referred to as immuno-thrombo inflammation is the consequence of disturbance of various biological pathways. Mechanisms include an inflammatory response to COVID-19, activation and damage of endothelial cells due to the binding of the spike protein of SARS-CoV-2 with ACE2 receptors on endothelial cells, platelet activation, aggregation, and deregulation of rennin-angiotensin system. Disturbances in these biological pathways leads to various long-term complications, including venous thromboembolism (VTE), disseminated intravascular coagulopathy (DIC), pulmonary embolism (PE), and arterial thromboembolism.

Endothelium is required for normal coagulation, and it is well recognized that endothelial cell destruction induces both intrinsic and extrinsic coagulation pathways, which in turn cause vessel occlusion. Inflammatory responses of COVID-19 such as high levels of interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF), and other inflammatory cytokines described as a "cytokine storm" alter fibrinolysis and natural anticoagulant pathway and activate endothelial cells, platelets, monocytes and tissue factors (Varga et al., 2020).

Another study (Levi et al., 2020) also found that severely affected COVID-19 patients have cytokine storm profiles characterized by high concentrations of proinflammatory cytokines, such as TNF-α and interleukins (IL-1 and IL-6), which activate coagulation pathway by inducing tissue factor (TF) and inactivating natural anticoagulant pathway. Endothelial cell injury due to this inflammation results in massive release of plasminogen activator, which induces fibrinolytic system, and high concentration of D-dimer and fibrin degradation product (FDP) is detected in patients with severe COVID-19.

Grover and Mackman, (2018) further stated that tissue factor (TF) is the high-affinity receptor for factor VIIa and is expressed on epithelial cells. The VIIA-TF complex activates the extrinsic pathway of coagulation by converting inactive protease factor X into active protease factor Xa. Under pathological conditions, inducible TF can trigger arterial and venous thromboses, leading to disseminated

intravascular coagulation. Another study of Cacciola et al., (2022) found that TF expression in monocytes occurs as a result of proinflammatory cytokine and thrombin production in moderate COVID-19 cohort. They also measured IL-6 and TNF-α levels, which reflect a higher inflammatory state.

Angiotensin-converting enzyme 2 (ACE2) functions as a receptor for SARS-CoV-2 (Varga et al., 2020). ACE2 is expressed on the endothelial cells of heart, kidney, intestine, liver, testis, adipose tissue, and central nervous system. They also stated that endothelial dysfunction causes vasoconstriction, inflammation, and a procoagulant state. This statement is well supported by the study of Escher, Breakey and Lämmle, (2020), who concluded that ACE2 present on endothelial cells are receptors for SARS-COV-2 and responsible for endothelial destruction and release of Von Willebrand factor (vWF) into blood which is stored in Weibel-palade bodies of endothelial cells.

Endothelial cell injury triggers primary hemostasis by activating events, such as platelet activation, aggregation, and adhesion to generate primary platelet plugs (Zhang et al., 2020). ACE2 and transmembrane serine protease 2 (TMPRSS2) are expressed on the platelet surface through which spike protein of virus binds via spike/ACE2 interactions, triggers the release of clotting factors and inflammatory mediators, and generates the leukocyte-platelet aggregates.

Derangement of hemostasis described by a study (Lippi et al., 2021) stated that primary hemostasis is triggered by the binding of SARS-CoV-2 on receptor ACE2 expressed on the surface of endothelial cells. Endothelial injury, followed by activation, adhesion, and aggregation of platelets, generate a platelet plug. This platelet plug is stabilized by fibrin generated by activation of coagulation cascade due to release of tissue factor (TF) from macrophages, and activation of macrophages occurs as a result of cytokine storm characterized by high interleukin values.

They also provided an overview of derangement of fibrinolysis, antiphospholipid antibodies, and renin-angiotensin-aldosterone system and concluded that COVID-19 has developed an immuno-thrombo-inflammatory thrombotic process which is most likely the result of numerous biological pathways, including endothelial damage, macrophage/monocyte activation, and neutrophil activation, which are all made worsened by continuous immobilization and the development of antiphospholipid antibodies.

It has been concluded that SARS-CoV-2 injured the vascular wall of blood vessels by binding with ACE2 expressed on the endothelium. Vascular injury causes vasoconstriction; high expression and secretion of Von Willebrand factor (VWF) promotes platelet aggregation at the site of vascular injury, reduces the expression of thrombomodulin and fibrinolytic heparin, and activates the coagulation cascade.

After the endothelial cells are injured, platelets stick to the vascular proteins, become degranulated, and release prothrombin activator, serotonin, adenosine diphosphate (ADP), and thromboxane A2 for their activation and mechanism of clot formation that start sequentially at the site of injury (Biswas et al., 2021).

Another report of Hess, Eldahshan and Rutkowski, (2020) revealed that angiotensin-converting enzyme 1 (ACE1) and angiotensin II (ATII) contribute to vasoconstriction, proinflammatory, and procoagulation effects. The renin-angiotensin system (RAS) is a hormone system in which angiotensinogen, produced by the liver, is cleaved into angiotensin I (ATI) by rennin secreted from juxtaglomerular cells in the kidney. ACE1 cleaves angiotensin I into angiotensin II. ATII induces tissue factor (TF) and plasminogen activator inhibitor-1 (PAL-1) expression and worsens endothelial function.

By directly cleaving ATII to angiotensin (1-7), ACE2 counteracts the harmful effects of ACE1 and ATII and protects endothelial function. During COVID-19 SARS-CoV-2, the spike protein interacts with ACE2, resulting in the depletion of ACE2. The unavailability of ACE2 favors the action of ACE1/AT2, which leads to a pro-inflammatory and pre-coagulation effect and contributes to endothelial dysfunction, tissue injury, and stroke.

Understanding COVID-19-associated Coagulopathy: A growing number of studies are showing that COVID-19 infection and coagulopathy are significantly correlated. Abnormalities in multiple coagulation measures, such as higher D-Dimer levels, extended prothrombin time (PT) and activated partial thromboplastin time (APTT) are indications of coagulopathy in COVID-19 patients. There exists a definite correlation between abnormalities in blood coagulation and the intensity of the COVID-19 disease. Patients who present with more severe manifestations of the illness tend to display more prominent deviations in their blood clotting measurements when compared to those with milder or moderate symptoms (Lin et al., 2021).

In particular, elevated levels of D-dimer have consistently been associated with greater disease severity and unfavourable clinical outcomes (Yao et al., 2020). This knowledge emphasises how crucial it is to keep updated on coagulation markers in COVID-19 patients in order to determine their risk of thrombosis and to direct treatment decisions. The coagulopathy linked with COVID-19 has complicated several pathophysiological processes.The hypercoagulable state seen in COVID-19 patients is thought to be caused by endothelial dysfunction, virally-induced proinflammatory cytokine release, and deregulation of the host immunological response. Furthermore, endothelial cell invasion by direct infection and coagulation cascade activation raise the risk of thrombosis (Iba, Connors and Levy, 2020).

Clinical Implications of COViD-19-associated Coagulopathy: Significant clinical consequences, such as an elevated risk of venous thromboembolism (VTE),

disseminated intravascular coagulation (DIC), and mortality are associated with coagulopathy in COVID-19 patients. Improving patient outcomes and preventing thrombotic consequences need the early detection and treatment of coagulopathy. Prophylactic and therapeutic anticoagulation have been used as strategies to reduce the risk of thrombosis in COVID-19 patients.

Challenges in Managing COVID-19-associated Coagulopathy: There are still a number of difficulties in managing COVID-19-associated coagulopathy, despite progress in our understanding of its etiology. Clinicians face difficulties in optimising anticoagulant medication due to variation in coagulation profiles, inconsistency in clinical presentation, and a lack of evidence-based guidelines. In addition, worries about bleeding complications linked to strong anticoagulation tactics emphasize the necessity of customized risk assessment and treatment. Studies available in the literature differ in their definitions of disease severity and the measurements used to evaluate outcomes, thereby emphasizing the necessity for standardized criteria to classify disease severity and uniformly assess clinical outcomes across various studies.

Further Direction and Research Implications: Moving forward, more investigation is necessary to clarify the best ways to treat COVID-19-associated coagulopathy. To support evidence-based practice, prospective studies assessing the effectiveness and safety of various anticoagulation regimens as well as the function of novel therapeutic medicines are required. Furthermore, coordinated efforts to standard treatment algorithms and diagnostic criteria would make it easier to provide high quality care to COVID-19 patients who are at risk of thrombotic problems.

Additionally, there is a need to analyze the coagulation profile of recovered COVID-19 patients so that early examination can be used for timely treatment, and early antithrombotic medication for prevention and treatment of thrombosis associated with COVID-19 will lead to enhanced results for recovered COVID-19 patients. The mechanisms that explain the link between blood coagulation abnormalities and disease severity are not fully understood, thus necessitating further investigations into the underlying pathophysiological pathways involved.

CONCLUSION

The literature review acknowledged close link between COVID-19 infection and coagulopathy with elevated coagulopathy markers such as D-Dimer levels, PT and APTT, especially in those patients who experienced severe manifestations of COVID-19 illness. It also underscores the clinical implications related to COVID-19 coagulopathy such as deep vein thrombosis (DVT), venous thromboembolism (VTE), disseminated intravascular coagulation (DIC) and increased mortality. However, there are several challenges in managing COVID-19-associated coagulopathy. Diverse coagulation profiles, uneven clinical presentations, and worries about bleeding side effects from anticoagulant medication are among challenges that clinicians must overcome. In summary, COVID-19-associated coagulopathy poses a serious clinical problem that affects patient outcomes and care. To optimize patient care and guide therapeutic strategies, a thorough understanding of the etiology and clinical symptoms of coagulopathy in COVID-19 patients is essential. In order to lessen the effect of coagulopathy on COVID-19 morbidity and mortality, research efforts must be sustained with the goal of filling up knowledge gaps and improving therapeutic approaches.

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Dental Age Assessment Using Demirjian and Cameriere's Methods in an Iranian Population During 2017-to 2018

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ABSTRACT

The present study aimed to compare the two dental age (DA) estimation methods of Cameriere and Demirjian among 6-14 year-old children in Tehran in 2017-2018. This cross-sectional analytical study involved 306 panoramic images from 153 girls and 153 boys. The DA of participants was estimated by Cameriere's and Demirjian's methods. The data were statistically analyzed by the paired sample t-test, repeated measures ANOVA, and the independent t-test. Finally, a formula suitable for Iranian society was developed based on the results of regression analysis. The mean age estimation error was +0.89 years for Demirjian's method (+0.86 in boys and +0.93 in girls) and -0.20 years for Cameriere's method (-0.20 in boys and -0.10 in girls). There was a significant difference between the DA calculated by Cameriere's and Demirjian's methods and the chronological age. There was no significant difference between Cameriere's and Demirjian's methods in this context. The formula developed in this study could estimate the age of participants with an accuracy of above +0.008 (+0.009 in boys and +0.006 in girls). However, the results indicated no significant difference between the proposed formula and Cameriere's method in the accuracy of age estimation.The accuracy of Cameriere's method was higher than that of Demirjian's method, but the formula proposed for Iranian society was more accurate than both of them. The Cameriere method underestimated and the Demirjian method overestimated the age.

KEY WORDS: CHRONOLOGICAL AGE, CAMERIERE'S METHOD, DEMIRJIAN'S METHOD, DENTAL AGE,

INTRODUCTION

Age estimation plays a major role in the diagnosis of endocrine problems 1, forensic dentistry, pediatric dentistry, and orthodontic treatment plans. Age estimation is used by orthodontists in specific orthodontic treatments and by pediatricians for evaluating teeth evolution (increased or decreased) in children with specific diseases. (Feijoo et al 2012, Butti, et al. 2009, Koshy and Tandon 1998, Hauk et al 2001, Graber et al 2012). Time plays an important role in the success of orthodontic treatment. Estimation of DA and skeletal age can help physicians determine the right time to begin treatments. Although there are skeletal and sexual indicators to determine growth rate, dental indicators are more commonly used for this purpose because they are less affected by nutritional and endocrine status, especially in children and adolescents, (Mani et al 2008, Molina et al 2020).

It has been shown that there is a relationship between dental calcification stages and skeletal development. DA is determined through Andrade et al (2019) methods: (1) assessing teeth growth in the oral cavity, and 2) examining tooth formation stages in X-rays. The first method is limited to children who haven't reached mixed dentition, and is affected by factors like premature tooth loss, ankylosis, or dental arch stenosis. Consequently, the second method is preferred for its broader applicability and reliability, (Baccetti et al 2005, Andrade et al 2019).

Developed in 1973, Demirjian's method is one of the most widely used methods in measuring dental development. Demirjian et al (1973) studied 7 permanent teeth on the left

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side of the mandible from 2928 panoramic radiographs of 3-16-years-old Canadian-French people and then developed a table of indicators and a conversion table. To solve the previous problems, Demirjian et al. (1973) increased the number of samples to 2047 boys and 2349 girls. They classified the course of dental development (from dental bud to completion) under 8 stages named A through H, (Demirjian and Goldstein 1976). Cameriere et al. (2006) proposed a novel age estimation method based on their study of 213 boys and 242 girls in Italy, which aimed to determine age by examining the relationship between age and the diameter of dental apices. The studies have shown that the age estimated by this method is very close to chronological age, (Rai et al 2006, Camerie et al, 2007, 2008).

Figure 1: Stages of dental development by Demirjian's method

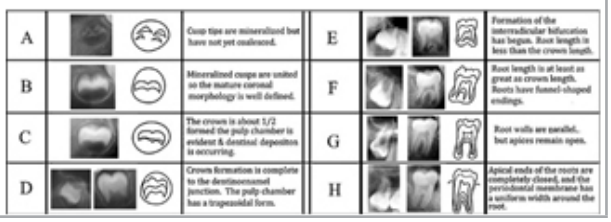
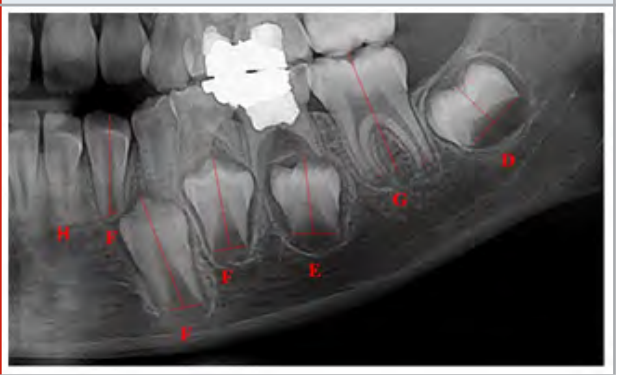
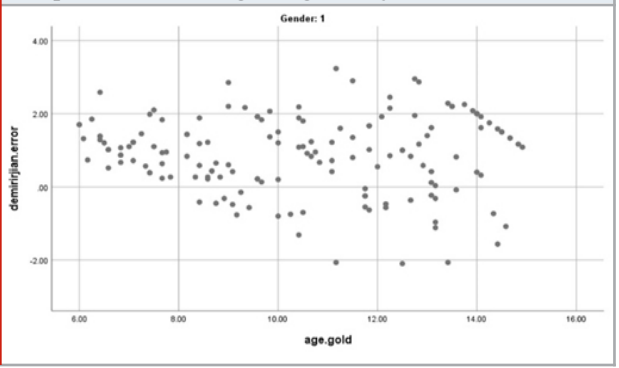


Figure 2: Measurement of dental length and width in Cameriere's metho

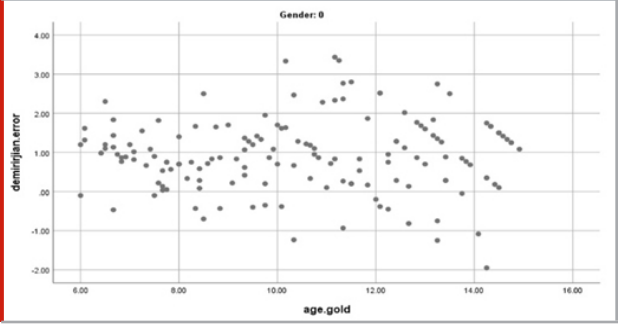


Scatter plot 1: Estimation error of Demirjian's method compared to chronological age in boys

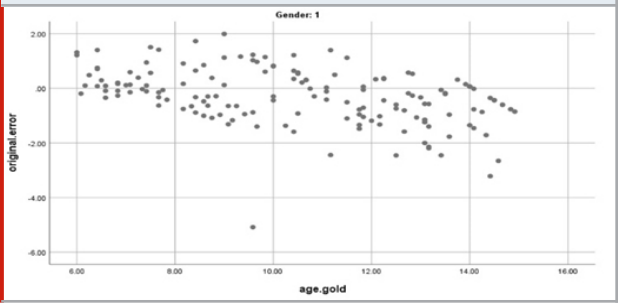


According to the literature, the course of dental development varies in different populations (even between different cities of a country) at different times. 16 These differences may be attributed to genetic or environmental factors such as socioeconomic status, nutrition, diet, and lifestyle or changes that occur over time in populations, (Marjatta et al 1988, Leurs et al 2005).The purpose of the present study was to compare dental age (DA) estimated through Cameriere's and Demirjian's methods with chronological age within the population aged 6-14 years in Tehran during 2017-2018. Additionally, the study aimed to develop a formula for this specific population using a regression equation.

Scatter plot 2: Estimation error of Demirjian's method compared to chronological age in girls



Scatter plot 3: Estimation error of Cameriere's method compared to chronological age in boys



MATERIAL AND METHODS

In this study, 306 panoramic radiographs of 6-14-years-old patients were collected from 4 oral and maxillofacial imaging centers in Tehran. The images were taken from March 2017 to June 2018. The inclusion criteria were as follows: Patients without developmental defects and systemic diseases affecting the growth. Panoramic images of high quality without any distortion. Patients with no missing teeth. Patients with a known date of birth. Lack of environmental factors affecting calcification such as inflammation or injury at the site. To begin, the chronological age of participants was determined by subtracting the radiography date from their date of birth, recorded with precision to two decimal places. Then participants were categorized into nine age groups, each spanning a one-year interval, with an equal number of participants across all age groups and both genders. Digital radiographs were taken using Cranex D (Finland, Helsinky, Sordex) and Planmeca (Finland, Helsinky, Planmeca).

Demirjian's method for age estimation was performed by 3 persons (two trained senior dental students and an oral and maxillofacial radiologist). In case of disagreement between the two observers, the radiologist's opinion was recorded as DA.According to Demirjian's method, 7 permanent teeth on the left side of the mandible were evaluated based on the course of dental development, which will be explained below (Figure 1). Then the table of indicators was used to give a number to each tooth considering its developmental stage (Table 1).

Finally, the 7 numbers obtained were added up to calculate the total maturity score, which ranges between 0 and 100. This score was converted into DA using the relevant tables and then compared with chronological age. To determine the error rate of Demirjian's age estimation method, the obtained DA was subtracted from chronological age. If DA was greater than chronological age, the values were reported with a positive mark. Otherwise, they were recorded with a negative mark.To estimate DA by Cameriere's method, images were opened in Adobe Photoshop-2018 to match them in terms of size and resolution.

All images were observed and evaluated in a 13.3-inch screen MacBook Air 2017 (1440 × 900) and Intel HD Graphics MB 1536, 6000 graphics. Then the images were measured by two observers. In case of significant disagreement, the measurement was repeated by a radiologist. In Cameriere's method, the 7 permanent teeth on the left side of the mandible were measured as explained in the following formula:

$$Age = 8.971 + 0.375\ g + 1.631\ X_5 + 0.674\ N - 1.034\ S - 0.176\ N.S$$

In single-rooted teeth (A_i, i=1…5), the distance between the inner wall of open-apex teeth was measured. In multi-rooted teeth, the mean distance between the inner walls of both roots (A_i, i=6,7) was separately calculated, and added up and then the length of the teeth was measured.

To neutralize the effects of magnification and X-ray angle, the dimensions were normalized by dividing them by the length of the tooth (L_i, i=1…7) ($X_i = A_i/L_i$ = 1…7). Moreover, the number of fully developed closed-apex teeth in each participant was counted and recorded as N0.

Finally, the normalized sum of the number of open-apex teeth (X_i), the number of open-apex teeth (S), the number of closed-apex teeth (N0), and gender (0 for girls and 1 for boys) were inserted into SPSS.To estimate age by Cameriere's method, all variables (N0, SN0, g, X_1, X_2. X_9) were defined and put in formulas proposed by Cameriere for Italian society. The obtained figure was subtracted from chronological age to calculate the estimation error.Based on the estimation error of Cameriere's method, a formula was developed for Iranian society. To this end, a regression equation was considered with age as the dependent variable and N0, SN0, S, X_1. X_7, and gender as independent variables. According to stepwise regression, variables N0, X_1, X_3, X7, SN0, and gender remained in the model, and the following regression line equation was obtained: Age = $9.309 + 0.636\ g - 3.852\ X_1 - 2.505\ X_3 - 1.007\ X_7 + 0.664\ N - 0.265\ SN0$

To reduce estimation error, the observers were unaware of the participants' age in both methods. In addition, the chronological age of participants was obtained by subtracting the radiography date from the date of birth.

Data analysis: Statistical analyses were performed in SPSS 24. Repeated measures ANOVA and the independent t-test were employed to compare the two studied methods in terms of the absolute value of estimation error and compare the absolute value of estimation error in girls and boys, respectively. Also, the DA obtained from Cameriere's and Demirjian's methods was compared with chronological age (overestimation or underestimation) by the paired sample t-test. *Demirjian. error: Demirjian's method error / ** Demirjian.error.abs: absolute value of Demirjian's method error,*Cameriere. error: Cameriere's method error / ** Cameriere.error.abs: absolute value of Cameriere's method error.

RESULTS AND DISCUSSION

The research involved the analysis of 306 panoramic images, comprising 153 girls and 153 boys aged between 6 and 14 years. The participants were divided into different age groups, each spanning a one-year interval, with each age group consisting of 17 boys and 17 girls.

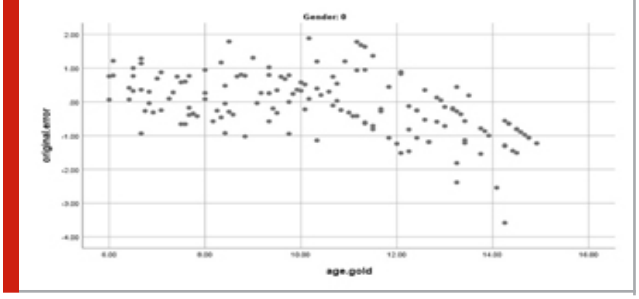
The results of repeated measures ANOVA for investigating the accuracy of Cameriere's and Demirjian's methods in the estimation of participants' chronological age are shown in Table 2. As shown in Table 2, Demirjian's method overestimated chronological age by 0.89 years, on average (0.86 in boys and 0.92 in girls). The absolute value of the mean estimation error for this method was 1.12 years (1.15 in boys and 1.09 in girls). Furthermore, Table 2 indicates that Cameriere's method underestimated chronological age by 0.20 years, on average (0.29 in boys and 0.10 in girls). The absolute value of the mean estimation error for this method was 0.75 years (0.79 in boys and 0.71 in girls).

Table 3 presents the results of the paired sample t-test to investigate the degree of overestimation or underestimation of age by Cameriere's and Demirjian's methods. The results showed that the DA estimated by both Cameriere's and Demirjian's methods was significantly different from chronological age ($p>0.05$). The results obtained from the formula proposed in this study are shown in Table 4. Based on Table 4, our formula underestimated chronological age by 0.0008 years, on average (0.0009 in boys and 0.0006 in girls). The absolute value of the mean estimation error for this formula was 0.71 years (0.76 in boys and 0.67 in girls).

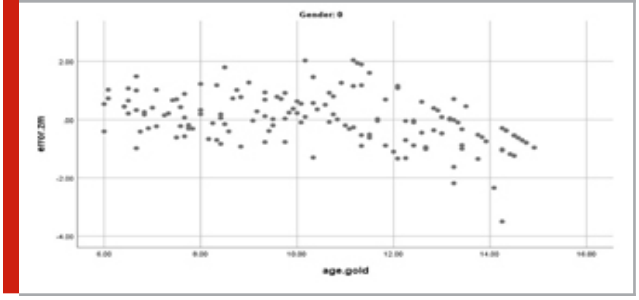
Table 5 shows the results of comparing Cameriere's and Demirjian's methods and our formula in terms of mean error and their absolute values for both boys and girls. Based on this table, the highest and the lowest estimate errors were related to Demirjian's method in boys and the

formula developed in this study in girls, respectively. The highest accuracy of Demirjian's method and Cameriere's method was observed in the age group 8-9 years and age group 7-8 years in both sexes, respectively. Moreover, the highest accuracy of the formula developed in this study was related to the age group 6-7 years in boys and 7-8 years in girls. The three above-mentioned methods have been compared with each other in the estimation error in scatter plots 1 through 6.

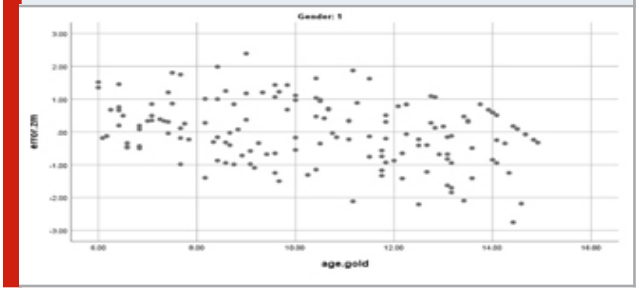
Scatter plot 4: Estimation error of Cameriere's method compared to chronological age in girls



Scatter plot 5: Estimation error of Mohammadi's(proposed formula) method compared to chronological age in boys



Scatter plot 6: Estimation error of Mohammadi's(proposed formula) method compared to chronological age in girls



When the absolute values of the estimation error of the three methods are compared, it can be concluded that there is a significant difference between Cameriere's method and Demirjian's method ($p<0.05$) and also between the formula developed in this study and Demirjian's method ($p<0.05$). By contrast, there was no significant difference between the formula developed in this study and Cameriere's method in this regard ($p>0.05$). The results of the independent t-test indicated no significant difference between boys and girls in terms of the absolute value of the estimation error of all methods ($p>0.05$).

Various methods with different accuracies have been proposed to evaluate the evolution of dental structure, such as Demirjian, Cameriere, Smith, Willems, and Haavikko, (Demirjan et al 1973, Cameriere, et al 2008, Haayiikko 1974, Smith 1991).

This study aimed to investigate the accuracy of Cameriere's and Demirjian's methods in DA estimation from panoramic radiographic images in the Iranian population and to propose a formula suitable for Iranian society. The results showed that the DA estimated by both Cameriere's and Demirjian's methods was significantly different from chronological age in the studied Iranian population ($p<0.05$). The results also indicated that the estimation error of Demirjian's method was higher than that of Cameriere's method. The mean absolute value of estimation error was 1.1 years in Demirjian's method and 0.7 in Cameriere's method. The estimation error of both methods was observed in both genders and all age groups ($p<0.05$).

Demirjian's method overestimated DA in both genders and all age groups, whereas Cameriere's method underestimated DA in all age groups of girls but overestimated in the age group 6-10 years. Cameriere's method followed no specific pattern, as it overestimated DA in age groups 6, 7, and 10 years but underestimated DA in other age groups.

Previous studies have reported that the estimation error of Demirjian's method compared to chronological age ranged between 0.13 and 0.97 years in girls and between 0.09 and 0.98 years in boys, (Grover et al 2011, Sakhdari, et al 2015, Wolf et al 2016, Pinchi et al 2016, Apaydeen et al 2018, Ginzelová et al. 2015, Mohanty et al 2019, Ali et al 2019).

However, some studies have shown that Demirjian's method underestimates DA, (Alqadi et al 2019, Lan et al 2019), as it ranges between 0.15 and 1.03 years in girls and between 0.04 and 0.89 years in boys (Table 6). It seems that the results of DA estimation by Demirjian's method do not follow a clear pattern in different populations. Most studies conducted on Demirjian's method in Iran reported that this method overestimates DA compared to chronological age, Javadinejad, 2015, Shaeikhi et al 2019), the latter conducted two similar studies on children aged 5-16 years in Babol and Rasht. Their results demonstrated that Demirjian's method underestimated DA by 0.04 years in Babol but overestimated DA by 0.02 years in Rasht compared to chronological age, (Shaeikhi et al 2012, 2013). This discrepancy can be attributed to ethnic, environmental, nutritional, and socioeconomic differences as well as differences in sample size and statistical analysis.

After Cameriere carried out a study on a large European population and developed a formula for DA estimation in 2006, different studies reported different results of age estimation by this method. 15, 22-24, 39, 42 Cameriere (2006) stated that the accuracy of his method was -0.11

years 12. In the present study, the mean accuracy of Cameriere's method in age estimation was obtained at -0.20 years (-0.11 years in girls and -0.29 in boys). Various studies have shown that the estimation error of Cameriere's method is less than that of chronological age. These studies have reported that the estimation error of Cameriere's method ranges between 0.08 and 0.96 years in girls and between 0.07 and 1.07 years in boys, (El Bakery 2010, Kumaresan 2014), (Table 6).

Table 1: Dental indices in each stage for girls and boys

Tooth		A	B	C	D	E	F	G	H
Boys	2nd molar	0.18	0.48	0.71	0.8	1.31	2	2.48	4.17
	1st molar	-	-	-	0.69	1.14	1.6	1.95	2.15
	2nd premolar	0.08	0.05	0.12	0.27	0.33	0.45	0.4	1.15
	1st premolar	0.15	0.56	0.75	1.11	1.48	2.03	2.43	2.83
	Canine	-	-	-	0.04	0.31	0.47	1.09	1.9
	Lateral incisor	-	-	0.55	0.63	0.74	1.08	1.32	1.64
	Central incisor	-	-	1.68	1.49	1.5	1.86	2.07	2.19
Girls	2nd molar	0.14	0.11	0.21	0.32	0.66	1.28	2.09	4.04
	1st molar	-	-	-	0.62	0.9	1.56	1.82	2.21
	2nd premolar	-0.19	0.01	0.27	0.17	0.35	0.35	0.55	1.51
	1st premolar	-0.95	-0.15	0.16	0.41	0.6	1.27	1.58	2.19
	Canine	-	-	0.6	0.54	0.62	1.08	1.72	2
	Lateral incisor	-	-	-	0.29	0.32	0.49	0.79	0.9
	Central incisor	-	-	1.83	2.19	2.34	2.82	3.19	3.14

Table 2: Mean, standard deviation, maximum, and minimum estimation error and their absolute values for Cameriere's and Demirjian's methods

	Number	Minimum	Maximum	Mean	Std. Deviation
Demirjian.error*	306	-2.10	3.43	0.8971	0.99261
Demirjian.error.abs**	306	0.03	3.43	1.1235	0.72537
Cameriere.error*	306	-5.08	1.99	-0.2017	0.96282
Cameriere.error.abs*	306	0.00	5.08	0.7554	0.62869

Table 3: Results of the paired sample t-test for determining the overestimation or underestimation of age by Cameriere's and Demirjian's methods and their p-values

	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		df	Sig. (2-tailed)
				Lower	Upper		
camereiere.age - chronological.age	-0.20165	0.96282	0.05504	-0.30996	-0.09334	305	0.000
demirjian.age - chronological.age	0.89706	0.99261	0.05674	0.78540	1.00872	305	0.000

It can be stated that previous studies have reported different accuracies for Cameriere's and Demirjian's methods; some studies have shown that Demirjian's method is more accurate than Cameriere's method, (Wolf et al 2016, Pinchi et al 2012, Timmins et al 2011), whereas other studies, like the present study, concluded that Cameriere's method is more accurate, (Timmins et al (2011), Javadidenaj 2015).

Some studies have stated that the accuracy of Cameriere's or Demirjian's methods is higher in a certain age group. For example, (Bagherpour et al. 2010, and Javadinejad et al.

2015, and Timmins et al 2011) showed that the accuracy of Demirjian's method was higher in age groups 9-13, 6-11, and 16 years, respectively. Timmins et al. (2011) also reported that Demirjian's method was more accurate for older ages. This discrepancy may be attributed to racial, socioeconomic, and nutritional differences as well as differences in sample size.A comparison of the absolute values of the mean estimation error in this study showed that the highest accuracy of Demirjian's method was observed in age groups 8-9 and then 7-8 years in both genders and the highest accuracy of Cameriere's method was related to the age group 7-8 years in both genders.

Table 4: Mean, standard deviation, maximum, and minimum estimation error of the proposed formula

	Number	Minimum	Maximum	Mean	Std. Deviation
Our Method.error*	306	-3.50	2.39	0.0008	0.91146
ABS.error. Our Method**	306	0.00	3.50	0.7193	0.55829

* Our Method.error: estimation error of the formula proposed in this study **ABS.error. Our Method absolute value of estimation error of the formula proposed in this study.

Table 5: Mean, standard deviation, maximum, and minimum estimation error of Cameriere's and Demirjian's methods and the formula proposed in this study and their absolute values for both boys and girls for a number of 153

	Girls				Boys			
	Minimum	Maximum	Mean	Std. Deviation	Minimum	Maximum	Mean	Std. Deviation
demirjian.error	-1.95	3.43	0.9291	0.93254	-2.10	3.23	0.8650	1.05135
demirjian.error.abs	0.03	3.43	1.0920	0.73355	0.03	3.23	1.1550	0.71813
error.Our Method	-3.50	2.04	0.0006	0.87000	-2.75	2.39	0.0009	0.95398
ABS.error.Our Method	0.00	3.50	0.6744	0.54690	0.03	2.75	0.7642	0.56769
Cameriere.error	-3.58	1.88	-0.1080	0.89360	-5.08	1.99	-0.2953	1.02174
Cameriere.error.abs	0.00	3.58	0.7170	0.54110	0.01	5.08	0.7939	0.70522

The accuracy of Cameriere's method in different age groups has been measured in different studies, (Galic et al 2010, Javadinejad et al. 2015, da Luz et al (2019). Consistent with the findings of the present study, Javadinejad et al. (2015) reported that Cameriere's method was more accurate in girls aged 6-11 years and boys aged 6-12 years.

In addition, Da Luz et al. (2019), Golsahi et al. (2015) and Rivera et al. (2017), showed that the highest accuracy of Cameriere's method was observed in people aged 8, 9, and 13 years, respectively. Galic et al. (2010) stated that the highest accuracy of Cameriere's method was related to the age of 15 years in boys and 12 years in girls, (Galic et al 2010). Available studies have reported that DA of boys is higher than girls in children aged under 8.5 years, whereas dental development is faster in girls in children aged 9-12 years. This can be attributed to the fact that girls reach puberty at this age, (Feijoo 2012, Halilah 2018).

In this study, the DA of boys aged under 9 years was about 0.2 years more than DA of girls of the same age. By contrast, the DA of girls was more than boys by 0.45 and 0.53 years in children aged 10 and 11 years, respectively. However, these differences were not statistically significant.

Previous studies have generally reported that both Cameriere's and Demirjian's methods underestimate DA at older ages and overestimate DA at younger ages, (Liversidge 2010, Guo, et al (2014). There are various reasons for DA underestimation at older ages. One of the main reasons is that all teeth have developed and there are a few teeth with non-developed roots or lately-developed roots in children of this age group. The number of developing teeth decreases as people grow older, and only the third molar remains non-developed by the age of 14 years, Liversidge 2010).

One of the reasons for the different results in different studies is the choice of different age ranges. According to available radiology references, since surgical and orthodontic treatments are not usually recommended in patients aged under 6 years (except for emergency cases), it is preferable to perform radiology and treatment at the same time, (White and Pharaoh 2014). When the apex of the distal root of the second molar is closed in children, the course of dental development is considered to be accomplished. Since the third molar is not investigated in most methods of DA estimation for children, these methods select the age of 14 years as the end of the studied age range. In the present study, the age range of participants was 6-14 years.

However, the findings of previous studies suggest that Demirjian's method can be used for age estimation before puberty. Accordingly, if the second molar, canine, and the first premolar teeth are at F, F, and E stages, respectively, it can be stated that one is in the pre-pubertal phase (equivalent to phases CS1 and CS2 in cervical vertebrae cervical maturation). When vertebral and dental development phases are compared, it can be also concluded that girls reach puberty at the age of 12 years, which is equivalent to Stage G in the second premolar, (Tafakhori et al 2016).

Table 6: A summary of previous studies conducted about the accuracy of Cameriere's and Demirjian's methods

Author(s)	Year of publication	Country	Accuracy of Demirjian's method in girls	Accuracy of Demirjian's method in boys	Accuracy of Cameriere's method in girls	Accuracy of Cameriere's method in boys
Rozylo et al. 60	2008	Poland	-1.03	-0.89		
Qudeimat et al.	2009	Kuwait	-0.67	-0.71		
Bagherpoor et al.	2010	Iran(Mashahad)	+0.25	+0.34		
El Bakary e al.	2010	Egypt			-0.26	-0.49
Sheikhi et al.	2011	Iran(Babol)	+0.04	+0.02	2	
Bagherian et al.61	2011	Iran(Rafsanjan)	+0.21	+0.15		
Lee et al.	2011	South Korea	+0.86	+0.64		
Ogodecu et al.	2011	Romania	+0.36	-0.04		
Javadinejad et al.	2012	Iran(Isfahan)	+0.47	+0.94		
Pinchi et al.	2012	Italy	+0.41	+0.68	-0.96	-1.07
Grover et al.	2012	India	+0.56	+0.66		
De luca et al.62	2012	Mexico			□0.63□	□0.52□
Abbesi et al.63	2012	Iran (Babol)	□0.05□	□0.72□		
Sheikhi et al.	2013	Iran(Rasht)	-0.1	+0.28		
Kumaresan et. al.	2013	Malaysia	+0.97	+0.97	-0.39	-0.44
Javadinejad et al.	2015	Iran(Isfahan)	+0.85	+0.90	-0.11	-0.27
Ginzelova et al.	2015	Czech Republic	+0.13	+0.09		
Wolf et al.	2016	Germany	+0.17	+0.16	-0.08	-0.07
Melo et al.	2017	Spain	+0.85	+0.85		
Apaydin et al.	2018	Turkey	+0.30	+0.31	-0.55	-0.60
Halilah et al.	2018	Germany			-0.38	-0.64
Wang et al.	2018	China	-0.62	-0.66		
Sobieska et al.	2018	Poland	-0.31	-0.31		
Kermani et al.64	2019		Iran(shiraz)	□1.47□	□0.85□	
Mohanty et al.	2019	India	+0.56	+0.66		
Alqadi et al.	2019	Yemen	-0.40	-0.73		
Moness Ali et al.	2019	Egypt	+0.32	+0.46		
Ranasinghe et al.	2019	Sri Lanka	+0.19	+0.19		
Da luz et al.	2019	Brazil			□1.05□	□1.08□
Lan et al.	2019	Croatia China			□1.19□ -0.72	□1.2□ -0.83
Pan et al.65	2020	China	|0.79|	|0.73|		
Karimi et al.66	2021	Kuwait	+0.33	-0.14		

Various methods of DA estimation are used to predict the beginning and completion of orthodontic treatments. In cases where there is a delay in tooth eruption, we can use the patient's radiograph and compare the conditions of each tooth with the standard conditions to predict the time of tooth eruption. Demirjian and Levesque stated that a tooth is about to erupt if it is at Stage G but it takes about one year to erupt if it is at Stage F. They also suggest that if a permanent tooth is at Stage F, the deciduous tooth covering it should be extracted., (Levesque et al (1980).

Chronological age estimation based on Cameriere's formula will have the least error in Iranian society when the obtained figure is added to 0.2. Moreover, to maximize the accuracy of Demirjian's method, 0.87 should be subtracted from the figure obtained from this formula.

Considering the estimation error obtained for Cameriere's formula in this study, a domestic formula was designed in this study based on Cameriere's formula to suit the Iranian population. The designed formula was as follows:

$$Age = 9.309 + 0.636\ g - 3.852\ X_1 - 2.505\ X_3 - 1.007\ X_7 + 00.664\ N - 0.265\ SN0$$

Compare the developed formula with Cameriere's formula:

$$Age = 8.971 + 0.375\ g + 1.631\ X_S + 0.674\ N - 1.034\ S - 0.176\ N.S$$

Where, the dental index was considered to be 5 (X_5), and teeth 1, 3, and 7 (X_1, X_3, and X_7) were replaced.

Some previous studies have developed population-specific formulas for age estimation. However, the accuracy of the developed formulas is not always more than that of Cameriere's method. Hallilah (2018) argues that this is due to the unequal number of samples in different age groups. This discrepancy was also observed in the present study despite the acceptable number and uniform distribution of samples in different age groups.When the formula developed in this study is compared with Cameriere's and Demirjian's methods, it can be concluded that the accuracy of the formula developed in this study was higher than that of the other two methods ($p<0.05$). Hence, researchers are recommended to use this formula in studies conducted on Iranian populations.

Some of the limitations of these methods that can cause differences in results are as follows: Those who have a missing tooth cannot be included in the study. Cameriere's method requires the measurement of open-apex teeth. This is difficult to do in teeth where the apex is closed. Conduction of studies on people of different age groups or genders can lead to a discrepancy in results. Nutritional, social, and economic issues can affect the course of dental development, (Timmins et al 2011). There is a difference between races and ethnicities in terms of the rate of dental development, (Shaikhi et al 2013).

Although the panoramic technique has many advantages, slight changes in the X-ray tube angle or patient position (the object is places a bit backward or forward in the focal trough) can cause dimensional changes in the resulting images, (Tafakhori et al 2016). Considering technological advantages, age estimation methods are recommended to be based on the use of cone beam computed tomography (CBCT), as this method has been used in recent studies, (Kazmi et al 2019, Molina et al 2020).

CONCLUSION

Both Cameriere's and Demirjian's methods are not highly accurate and the DA estimated by them is significantly different from chronological age. However, the study findings revealed that Cameriere's method was more accurate than Demirjian's method. The formula designed in this study for Iranian society was more accurate than both of the above-mentioned methods. All three methods had almost the same accuracy in both genders. However, Cameriere's method underestimated the age, and Demirjian's method overestimated the age compared to the chronological age.

Conflict of Interest: Authors declare no conflict of interest.

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Identification and Characterization of Amylolytic Bacteria from Agro-industrial Waste Water

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ABSTRACT

Introduction: Looking for novel amylases needed for industrial processes may benefit from the screening of microorganisms with high amylase activity. Microbial enzymes are widely used in industrial processes due to affordable cost, significant production, chemical stability, environmental protection, plasticity, and widespread availability. Since Purba Bardhaman contains numerous rice processing plants and agro-industrial waste disposal locations, water samples were gathered for the study. Material & Methods: Amylase-producing bacteria were isolated, identified and revealed from this work to add new knowledge into the field of science. Seven bacterial isolates were isolated, and based on appearance of zone of hydrolysis in starch agar plates was selected for further study. The isolate was Gram-positive, spore-forming rods. Depending on 16s rRNA gene sequences with 99.79% similarity the isolate was identified as *Brevibacillus parabrevis*, efficient amylase producers with an excellent yield throughout the solid-state fermentation technique. The isolate demonstrated maximum enzyme activity of 350 U/mg at 5% substrate concentrations. This piece of work focuses on finding out whether agro-industrial waste water could be a better source for amylolytic bacteria.

KEY WORDS: AGRO-INDUSTRIAL WASTE WATER, AMYLOLYTIC BACTERIA, BIOCHEMICAL CHARACTERIZATION, SCREENING.

INTRODUCTION

Microbial population can be manipulated to yield enzymes which are commercially important in organic compound synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation. Microorganisms can easily be targeted as economical source for industrial enzyme production. Use of cheap, easily accessible wastes, such as agro-industrial waste, as a novel substrate for production and synthesis of amylase for industrial use is an ongoing effort that helps to address pollution issues. Amylase, an enzyme, is needed for the catalytic degradation of starch into its monomeric elements, of which glucose is the smallest, (Logeswaran et al.2014, Saha et al.2019).

Looking for novel amylases needed for industrial processes may benefit from the screening of other microorganisms with high amylase activity. Microbial production of amylase is more fruitful than other sources like plants or animals, because of short growth period, biochemical diversity and simplicity with environmental and genetic manipulation could improve the capacity for enzyme synthesis, Mishra and Behera (2008), Saha et al (2019). In order to substitute enzymes, which are typically extracted from complex eukaryotes because to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being explored, (Bole et al.2013). Microbial enzymes are widely used in industrial processes due to affordable cost, significant production, chemical stability, environmental protection, plasticity, and widespread availability, (Mishra and Behera 2008, Deb et al.2013, Burhan et al.2003).

Approximately 25% of the enzyme market is comprised of amylase enzyme. Fungi and bacteria are the best choices of the source because they are very economical with high production rate and can be genetically engineered for the desired quality and quantity of amylase production, (Islam et al 2017). In biotechnological applications ranging from food fermentation, detergent, pharmaceutical, brewing, and textile to paper industries, amylases perform a significant role, (Kathiresan and Manivannan 2006). Low-cost amylase production is necessary to meet the greater demands of these sectors, (Saxena and Singh 2011). Earlier, amylase production has been studied using submerged (SmF) and solid-state fermentation (SSF), Perez-Guarre et al. (2003), Saxena and Singh (2011).

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However, as the elements of a synthetic medium are very costly and uneconomical, they must be substituted with agricultural and industrial waste products, which are thought to be a viable source for microbial populations that produce enzymes and are more readily available. In this present experimental work, isolation and screening of amylase producing microbes has been attempted with samples collected from rice processing units in Bardhhaman, West Bengal. Since these areas are rich in rice processing units and agro-industrial waste, therefore, data of this work might contribute new insights into finding cheaper sources for amylase producing microorganisms. This process was achieved by stepwise activities including isolation of α-amylase producing microorganisms from the waste water sources; selection and identification of the most potent isolate for in-vitro enzyme production while utilizing the solid media; and optimization of culture conditions affecting α-amylase production by the selected isolates.

MATERIAL AND METHODS

Isolation of microorganism: Waste water sample was collected from agro-industrial dump of rice processing unit of Purba Bardhaman, West Bengal India. The study region is situated at 23°15'15.3"N, 88°01'50.9"E latitude and longitude. Water sample was collected by using sterile containers and was stored in 4ºC for subsequent analysis. As the water sample was collected from the dump area of rice processing unit, there might be presence of amylolytic microorganisms so selective media along with basic media was used for isolation of microbes. 0.1 mL of every specimen has been put into nutrient agar plates from the container. (Beef extract 10g/L; Peptone 10 g/L; Sodium Chloride 5 g/L; Agar 15 g/L; pH 7.2±0.1) as well as starch agar plates (Beef extract 3 g/L; Peptone 5 g/L; Soluble starch 10 g/L; Agar 15 g/L; pH 7.2 ± 0.1) in triplicate. After that, the microbial culture was spread using spreader and maintained for 24 hours at 37°C. Colonies were cultured on the proper medium after the incubation time to generate pure isolates, and were then kept at 4°C for more study.

Screening of microorganism: The pure microbial culture obtained was cultured on starch agar plates to authenticate whether the microbial culture obtained was amylolytic bacterial species. After 24 hours of incubation, the plates were immersed with iodine solution for 30 seconds. Clear zones encircling the growth of microbes were considered to be amylase producers.

Characterization and identification of amylase producing isolates: The isolates were assessed by the gram reaction and colony morphology, respectively using the methods of Collins and Lyne. Collins et al.(2004), Sinha. (2010). Additionally, other biochemical assays such as IMViC Test, Urease test, and Starch hydrolysis tests, sugar fermentation, nitrate reduction test were from carried out to characterize isolates in terms of their biochemistry. As the sample was collected from waste water so coliform test was also carried out. 16s rRNA gene sequences was also carried out for authentication of the species and genera of the microbes. Saitou and Nei, (1987).

Standardization of substrate concentration and incubation period for enzyme production: Substrate concentration for enzyme production was optimized using different concentrations of starch (1.0, 2.5, 5.0, 7.5 and 10.0%) with different incubation period of time (24, 48, 72, 96 and 120 h) in the production medium.

Amylase production by using Solid State Fermentation (SSF): After standardization of the substrate concentration and incubation period, the SSF process was carried out using rice husk. Substrate bed (Rice Husk: 10 g, Starch: 0.5 g, KH_2PO_4: 0.2 g, NaCl: 0.25 g, $MgSO_4$, $7H_2O$: 0.02 g, $CaCl_2$:0.1 g, $(NH4)_2SO_4$: 0.1 g) was prepared and transferred into 250 mL capacity Erlenmeyer flasks. Moisture content was adjusted with 20 mL de-ionized water and autoclaved and allowed to cool to room temperature. 10 mL of 24 h old microbial cultures was added into the substrate medium and incubated for 96 h, (Tsegaye et. al.2014).

Extraction of crude enzyme: The extracellular enzymes from the fermented substrate were extracted using phosphate buffered saline (50 mL) after proper agitation on a rotary shaker at 120 rpm for 45 minutes. The content was filtered and squeezed through a cotton cloth. The filtrate was used as the crude enzyme, Tsegaye et. al. (2014).Determination of enzyme activity: Activity of α-amylase was determined by 3,5 Di-nitro salicylic acid (DNSA) method, Miller (1959) using Potato Starch as substrate and Sodium Phosphate buffer (pH-6.9) as the incubation medium at 37°C of incubation temperature. Total protein content was determined, Lowry et al (1951).

RESULTS AND DISCUSSION

Isolation & Screening of Microorganism: As a result of the preliminary screening, many isolates having the capacity to synthesize amylase at different levels were identified. Seven potential microbial isolates were obtained from the waste water samples were marked as Sp1-Sp7 depending upon their growth on nutrient agar as well as clear zone formation in starch agar media after reacting with gram's iodine solution (Table-01) and this outcome was consistent with the findings of Hmidet et. al. (2009) from the starch hydrolysis test, Sp1 was selected for further investigation.

Table. 1: Bacterial Isolates with their clear zone on Starch agar

Bacterial Isolates	Clear Zone(mm)
Sp1	25.0 ± 0.5
Sp2	10.0 ± 0.1
Sp3	17.0 ± 0.2
Sp4	15.0 ± 0.6
Sp5	8.0 ± 0.1
Sp6	4.0 ± 0.9
Sp7	1.5 ± 0.3

Characterization of amylase producing isolates

Colony Morphology: The most potent isolate that showed highest starch hydrolyzing ability was selected for further characterization. The isolate was characterized based on colony growth feature and microscopic observation (magnification of 100X and 400X) to distinguish their respective genera. Sp1 demonstrated a regular form, with color and rod shape of colony morphology (Table-02).

Table. 2: Colony morphology of the Isolates

Bacterial Isolates	Clear Zone(mm)
Characteristics	Bacterial Species (Sp1)
Configuration	Round
Margin	Entire
Elevation	Raised
Surface	Smooth
Density	Opaque
Pigmentation	White
Gram reaction	Positive (+ Ve)
Cell morphology and spore	Rod shaped and Oval

Effect of different physico-chemical factors: Effect of different physico-chemical factors like temperature, pH and salt concentration (NaCl) was also observed. As per observation the microbial culture is adaptable to a vast range of temperature from 20ºC-45ºC with optimum growth at 37ºC. The isolated microbes are capable to grow even in lower salt concentration (2%) even at lower water activity i.e. xerophile in nature.

Biochemical Characterization: Based on the biochemical characterization (Table-03) isolate may be *Brevibacillus* sp. Depending on the isolate's initial screening, these genera might be suitable for commercial applications, which the previous report corroborated by Ashwini et al. (2011).

16s rRNA gene Sequencing: The best amylase producing bacteria was isolated and identified by amplification and sequencing of its 16s rRNA full length coding gene, following the comparison of the obtained sequence with the NCBI database using the BLAST tool. The result showed that the selected stain was closely to Brevibacillus gene and in particular to the species parabrevis with 99.79% similarity. Therefore, the newly isolated strain was named as *Brevibacillus parabrevis.* 18109.

Standardization of substrate concentration & Incubation period for enzyme production: Fermentation conditions need to be optimized, especially with regard to physical and chemical characteristics, Wenster-Botz (2000). Amylase activity generally increased while starch concentration increased from 1.0% to 10.0%. In this investigation, 5% of starch content provided the maximum activity (Figure-02) at different incubation time. The present finding is also endorsed with previous investigations on amylase activity, as reported by Oyeleke and Oudwole (2009). At 96 hours into the fermentation procedure, the isolate's amylase activity reached its peak, after which it began to drop (Figure-02). *Bacillus subtilis* and *Bacillus* sp. DLB9 has also exhibited comparable outcomes, Shyam et al.(2013).

Figure 1: Phylogenetic tree of strain 18109 based on 16S rRNA gene sequences.

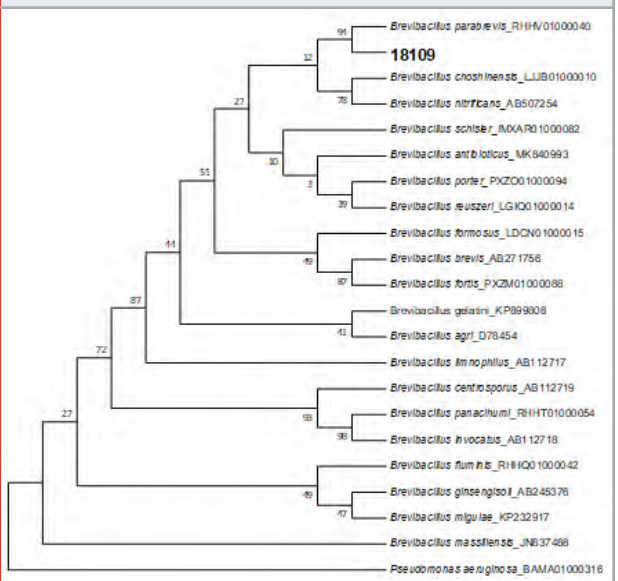


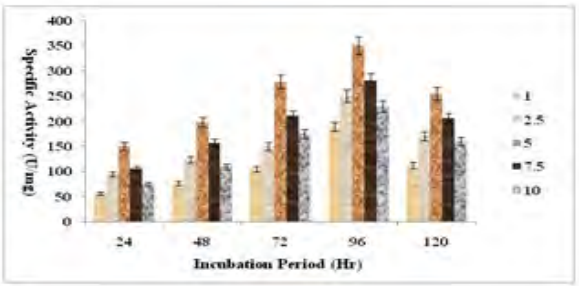
Table. 3: Biochemical Characteristics of Bacterial Isolate

Biochemical tests		Bacterial Isolate (Sp1)
Growth on Mac Conkey agar medium		-
Indole test		+
Methyl red test		-
Voges Proskauer test		-
Citrate hydrolysis		-
Casein hydrolysis		-
Starch hydrolysis		+
Gelatin hydrolysis		-
Nitrate reduction		-
Catalase		-
Esculine hydrolysis		+
H2S gas production		-
Acid production from carbohydrates	Dextrose	-
	Fructose	-
	Sucrose	-
	Lactose	-

+ Positive: - Negative

Determination of potential enzyme activity: The crude enzyme isolated from Sp1 showed the maximum specific activity of 350 U/mg, the current results are consistent with past reports obtained from Bacillus species, Bukhari and Rehman (2015).

Figure 2: Standardization of Substrate Concentration & incubation Period



The experimental piece of work was focused on obtaining microbial isolates from agro-industrial waste water having the ability to produce amylase. Many microbial species have already been identified as good amylase producers. Studies of amylases from bacteria and fungi are well available but using a cheaper resource for microbial isolation has not been documented. In our study, seven microbial isolates were identified from the five different waste water samples collected from the rice processing units. Though most of the isolated microorganisms are potent producer amylase but depending upon the starch breaking capacity of amylase produced by the microorganisms on starch agar media, Sp1 was selected for further investigation. Being neutrophilc in nature the microbe is adaptable to live in an environment where the hydrogen ion concentration is at equilibrium i.e. thrives in a relatively neutral pH, in the range of pH 5-9.

Even in lower salt concentration, the isolated microbe is capable to grow, which indicates its xerophilic nature. With 99.79% similarity, the isolated microbial stain was closely related to the Brevibacillus genus and in particular to the species parabrevis, potential candidates with several industrial applications for amylase production. Generally, the amount of amylase activity increased as the starch concentration rose from 1.0% to 10.0% with maximum activity at 5% concentration, but when excludes 5% concentration, amylase activity decreased. This might be because the isolates have the ability to metabolize starch within a short amount of time after the concentration was raised. The isolate showed maximum amylase activity at 96 h. The suppression and presence of other byproducts in the fermentation medium as well as a reduction in nutrients may be the causes of the amylase activity decline after 96 hours, Haq et al (2010), Gebreyohannes (2015).

CONCLUSION

The present study attempts to explore the potential of indigenously microbial isolates from easily available cheap source that are capable to produce amylases. The current findings bring about a decision that the agro-industrial waste water possesses the potential to be a source of amylase-producing microbes that might be used to create highly effective industrial amylases. The present study is the first-time report on the capability of alpha amylase producing activity by *Brevibacillus parabrevis*. The isolated bacterial species that showed higher amylase activity can be characterized and exploited further for various useful industrial applications.

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On the Use of Nano Formulation Techniques in Improving Drug Delivery Syatem

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ABSTRACT

Nanoparticles are a revolutionary medication delivery technology, as we all know. They have several positive impacts, such as the drug's efficacy and safety. We enumerate its efficaciousness during drug distribution in this review. One of the methods for more precisely delivering pharmacological substances to the intended tissue while lowering the total dosage and possible harmful side effects is drug nanoformulation. They may function as carriers of various active medicinal ingredients into a particularly body regions, or they may be therapeutic agents in and of themselves. As a truly multidisciplinary field of study, nanotechnology has benefited greatly from the contributions of chemists, physicists, biologists, and pharmaceutical scientists in the development of novel therapeutic and diagnostic approaches. The application of nanotechnology has advanced non-invasive imaging, nutraceutical delivery, cancer and HIV/AIDS treatment, and more. This review makes it clear that the use of nontechnology in medicine and drug delivery has created new avenues for individualized and secure treatment options. In the end, researchers are able to administer medications for longer periods of time with less frequent doses (sustained release), higher precision, and penetration in difficult-to-access tissues through the alteration of molecular size and surface features.

KEY WORDS: NANOPARTICLES, LIPOSOMES, NANOFORMULATION. DRUG DELIVERY,

INTRODUCTION

Polymeric particles made of synthetic or natural polymers, known as nanoparticles, are spherical in shape. Their sizes vary from 10 to 500 nm. These particles offer a wide range of possible uses due to their spherical form and high surface area to volume ratio. Nanoparticle size and surface characteristics have been studied to improve bioavailability, reduce clearance, and boost stability. By regulating these properties, the medication can now reach bodily tissue that might not have previously been reachable. Nanoparticles are divided into several categories based on their size, shape, and material qualities (Haleem et al., 2023).

Furthermore, nanoparticles can be hard (such as titania [titanium dioxide], silica [silica dioxide] particles, and fullerenes) or soft (such as liposomes, vesicles, and nanodroplets). The classification of nanoparticles often relies on their use, such as in diagnosis or therapy vs fundamental research, or it may be connected to how they were generated. They have also aided in the development of new techniques of administering treatment, such as giving local warmth (hyperthermia), limiting vasculature to sick tissues and tumors, and transporting medication payloads (Al-Abduljabbar & Farooq 2023).

Magnetic nanoparticles have been used to trace the progression of cancer along lymph nodes in place of radioactive technetium. The nanoparticles function by taking advantage of the contrast change caused by microscopic particles of superparamagnetic iron oxide in magnetic resonance imaging (MRI). Such particles can also be utilized to eliminate tumors by hyperthermia, which involves heating and destroying tissue on a small scale using an alternating magnetic field (Crintea et al., 2022).

Nanoparticles can be created to improve fluorescence imaging, positron emission tomography (PET), or ultrasound pictures. These strategies often need the nanoparticle's

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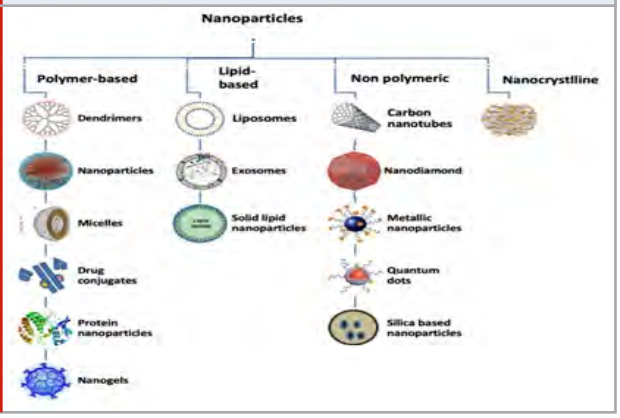
ability to recognize a certain cell or disease condition. The medication might be delivered by a nano capsule or a liposome, or it could be delivered in a porous nano sponge structure and then kept in place by bonding at the targeted spot, allowing for delayed drug release. The creation of nanoparticles to help with medicine delivery to the brain by inhalation offers great potential for the treatment of neurological illnesses such as Parkinson's, Alzheimer's, and multiple sclerosis (Jain et al, 2018).

Figure 1: Types of Nanoparticles.



Nanoformulations: Nano formulation of drugs is one strategy to deliver pharmaceutical agents more precisely to the targeted tissue and reduce the overall dose and potentially toxic side effects (Choi et al., 2023). Types of nano formulations: Nanocrystal: Nanocrystals have been utilized to deliver insoluble medicines like paclitaxel. PEGylation is a critical idea that extends the circulation duration of the nanocarrier system and enhances medication therapeutic outcomes (Sun et al., 2008). Nanocapsule: This has the potential to increase medication stability and bioavailability. Peptides, hormones, proteins, enzymes, medicines, metabolites, or reporter molecules may be protected from biological and chemical degradation using nano capsules (Janeth et al., 2017).

Nanospheres: Nanospheres are used in anti-wrinkle creams, moisturizing creams, and anti-acne nanoparticle creams. Nanospheres are utilized to transport active ingredients deeper into the skin, as well as to preserve the active component from enzymatic or chemical destruction or to provide a regulated release. In the case of scents, this delivery mechanism was found to extend active release (Prieto et al., 2017).

Nanosponges: They can solubilize weakly water-soluble medicines, resulting in extended release and improved medication bioavailability. The two primary therapeutic applications for nanosponges are targeted drug delivery (ensuring that the medicine reaches the target cells in the body, such as cancer cells) and enhanced drug delivery, which allows for improved physical qualities of pharmaceuticals (e.g., solubility)Nanoprecipitation: This technique involves quickly injecting a drug solution into an aqueous phase after it has been dissolved in a water-miscible organic solvent. Drugs precipitate quickly in aqueous media, forming nanoscale drug particlNano Formulation Based on Emulsions: Preparing an oil-in-water or water-in-oil emulsion and then letting the solvent evaporate to produce nanoparticles is known as solvent evaporation. [16] It includes phase inversion and spray drying (Sun et al., 2022).

Coacervation: In this method, a polymer solution is phase separated into a coacervate phase, which contains the medication. Upon solidification of the coacervate phase, nanoparticles may develop.

Electronspinning: Electrospinning is mainly used to manufacture nanofibers, but with the right formulations, it can also be utilized to produce nanosized particles.

Technology of Supercritical Fluids: Supercritical **Antisolvent Process:** To precipitate nanoparticles from a solution, antisolvents such as carbon dioxide are employed at supercritical temperatures. [20] Supercritical Fluid.

Extraction of Emulsions: In order, to extract nanoparticles from an emulsion, supercritical fluids must first be generated.

Nano carriers used in nanoformulation: Materials known as nano carriers are made with the purpose of encapsulating and delivering medicinal medicines, imaging agents, or other payloads in a precise and regulated way. These carriers play a crucial role in nanoformulations, improving medication stability, bioavailability, and solubility while frequently enabling tailored administration (Marianna Foldvari 2010). The following are a few typical nano carriers found in nanoformulations.

Liposomes: Lipid bilayers form the spherical vesicles known as liposomes. In their lipid bilayers or aqueous core, they can contain hydrophilic or hydrophobic medications, respectively. Liposomes can be used for a variety of medication delivery applications because they are biocompatible and adaptable (Zhang et al., 2018).

Polymeric nanoparticles: Biocompatible and biodegradable polymers are used to create polymeric nanoparticles. They can be made to release medications gradually or under strict supervision. Chitosan nanoparticles and poly (lactic-co-glycolic acid) (PLGA) nanoparticles are two examples.

Micelles: Made up of amphiphilic molecules, micelles are self-assembling structures. When these molecules are present in concentrations higher than their critical micelle concentration (CMC), they form. Drug distribution can be improved when hydrophobic medications are dissolved in the center of micelles.

Nanocapsules: Having a core-shell structure, nanocapsules are nanoscale capsules. Drugs can be accommodated in the core, while proteins, polymers, or lipids are frequently found in the shell. It is possible to encapsulate both hydrophobic and hydrophilic molecules using this architecture.

Dendrimers: Having a distinct structure, dendrimers are highly branching macromolecules. Their size and surface

functionality can be precisely controlled during their synthetic process. Drugs or imaging agents are frequently encapsulated inside of dendrimers.

Solid Lipid Nanoparticles: Solid Lipid Nanoparticles (SLNs) are room-temperature, lipid-based nanoparticles in a solid state. In comparison to conventional liposomes, they provide better stability and regulated release. Drugs can be shielded from deterioration by the lipid matrix. Protein-based.

Nanoparticles: Drug delivery nanoparticles can be formed from proteins, such as albumin or gelatin. These protein-based carriers can be engineered to have particularly targeting characteristics and are biocompatible.

Carbon nanotubes: Therapeutic compounds can be carried via carbon nanotubes, which are cylindrical structures with special features. Functionalized carbon nanotubes can be used as delivery systems for different payloads, such as imaging agents or drugs (Ganesh et al., 2015).

Metal nanoparticles: As carriers, metal nanoparticles derived from gold, silver, or iron oxide can be employed. Their surfaces can be functionalized for drug loading or targeting, and they may possess special features.

Cyclodextrins: Cyclodextrins are cyclic oligosaccharides that have ability to combine with hydrophobic medications to form inclusion complexes that increase the solubility of the former. They can serve as drug delivery vehicles, particularly for medications that are not very soluble in water (Patel et al., 2020).

Applications of nano formulation: Applications for nanoformulations can be found in many different domains, and they provide benefits like focused therapy, increased therapeutic efficacy, and better drug distribution. It includes1) Drug Delivery 2) Targeted Drug Delivery 3) Sustained Release 4) Cancer Therapy 5) Imaging and Diagnostics 6) Vaccines 7) Gene Delivery 8) Cosmetics & Personal Care 9) Agriculture 10) Food and Nutraceuticals 11) Wound Healing 12) Environmental Remediation To guarantee safety, scalability, and regulatory compliance in these applications, however, further research is necessary.

Methods used to improve drug delivery in nano formulation: One of the most important facets of pharmaceutical research and development is enhancing medication delivery. A range of techniques and tools are used to improve medication delivery's effectiveness, safety, and specificity. Here are some essential techniques for enhancing medication delivery.

Nanotechnology: Using nanoscale carriers to encapsulate medications, such as liposomes, micelles, polymeric nanoparticles, and dendrimers. This improves stability and solubility and enables tailored distribution.

Systems of Lipid-Based Delivery: Lipid vesicles known as liposomes are capable of encasing medications that are hydrophilic or hydrophobic. They enhance the stability and solubility of drugs and can help with targeted distribution. Lipid-based nanoparticles with regulated drug release and improved bioavailability are called solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs).

Delivery Systems for Polymers: Polymeric Nanoparticles: Nanoparticles for controlled medication release can be made from biodegradable and biocompatible polymers and targeted delivery.

Polymeric Micelles: Amphiphilic block copolymers self-assemble to generate self-assembling structures that improve the solubility of hydrophobic medicines.

Drug Pairs: Prodrug Design: The process of chemically modifying pharmaceuticals to produce prodrugs that, in their original form, are inactive or less active. In vivo activation enhances medication delivery and stability. Drugs and antibodies are linked to create antibody-drug conjugates (ADCs), which are then specifically delivered to target cells, such cancer cells. Encasing medication particles in microspheres or microcapsules to prevent deterioration and enable regulated release is known as microencapsulation.

Targeted Administration of Medicines: Active targeting is the process of delivering drugs to the intended location on cells by utilizing ligands, such as peptides or antibodies, to target particular receptors. Using the enhanced permeability and retention (EPR) effect in tumors, where leaky blood arteries allow nanoparticles to enter the body, is known as passive targeting. Using the increased permeability and retention (EPR) effect in tumors—where leaky blood arteries enable nanoparticles to aggregate preferentially in malignant tissues—is known as passive targeting.

Microneedle Technology: Transdermal drug delivery by microneedles allows for regulated release of medication by avoiding the epidermal barrier.

Electrospinning: This technique produces nanofibers that are used in tissue engineering, medication delivery, and wound healing.

Ultrasound-mediated delivery: The process of using ultrasound to improve drug penetration into tissues or cells, is referred to as ultrasound-mediated delivery.

Microfluidics: Using microfluidic devices to carefully manage the formulation process to produce nanoparticles or microcapsules with desired qualities is known as microfluidics.

Magnetic Drug Delivery: To improve targeted drug delivery, magnetic nanoparticles are guided to precise places using magnetic fields. Creating responsive systems that release medications in response to particularly stimulus, such as pH, temperature, or enzyme activity, is known as "smart drug delivery."

Routes of administration for nanoformulation: The drug's properties, the intended site of action, and the

intended therapeutic outcome all influence the delivery method selection.

The following are some typical medication delivery pathways for nanoformulations: Oral Administration, Intravenous Administration, Transdermal Delivery Intramuscular and Subcutaneous Injection Inhalation, Intrathecal and Intraventricular Administration, Intraperitoneal Administration, Ocular Delivery, Nasal Delivery, Delivery via Vagina and Rectal, Intradermal Delivery& Intraperitoneal Delivery. The advantages and disadvantages of each administration route are taken into consideration, while designing nanoformulations in order, to maximize drug delivery for certain therapeutic uses.

Factors that improve drug delivery in nano formulation: By using nanoscale carriers to address issues with drug solubility, stability, and targeted distribution, nanoformulations aim to improve drug delivery. The following elements influence how well drugs are delivered in nanoformulations.

Greater Surface Area: When compared to traditional formulations, nanoformulations offer a noticeably larger surface area. Better interactions with biological systems are made possible by the increased surface area, which enhances medication distribution and absorption.

Better Solubility: Hydrophobic medications' poor solubility is addressed via nanoformulations. Drug solubility and bioavailability are improved when drug particles are reduced to the nanoscale because this improves the effective surface area exposed to the surrounding medium.

Improved Bioavailability: Rapid drug absorption and distribution are made possible by the tiny particle size and larger surface area of nanoparticles, which enhances bioavailability. This is crucial for medications whose oral bioavailability is limited

Long-term Sustained and Controlled Drug Release: Drugs can be released over an extended period of time with the use of nano formulations. This controlled release profile enhances patient compliance, lowers adverse effects, and maintains therapeutic medication levels.

Targeted Drug Delivery: Certain tissues or cells can get drugs in a targeted manner thanks to nano formulations. While passive targeting can be accomplished by the increased permeability and retention (EPR) effect in some pathological circumstances, such as tumor tissues, active targeting is facilitated by surface modifications using ligands or antibodies.

Protection of Drugs: Liposomes and nanoparticles are examples of nanocarriers that can shield pharmaceuticals from enzymatic or adverse environmental degradation. During transportation and storage, this protection improves the stability of medications.

Better Cellular Uptake: Drugs that have trouble crossing cell membranes can benefit from nano formulations, which can improve cellular uptake. It is possible to use a variety of methods, such as receptor-mediated endocytosis, to help drugs enter target cells.

Decreased Side Effects: In nano formulations, targeted medication administration and controlled release help to minimize off-target effects and lower systemic toxicity. This is especially helpful for cancer treatment and other illnesses where accurate medication localization is essential.

Biocompatibility: To guarantee that nano formulations are compatible with biological systems, biocompatible materials are frequently used in their creation. This lowers the possibility of negative reactions and raises the medication delivery system's safety rating.

Customized Surface Properties: By altering their surface, nanoparticles can be made to exhibit particularly characteristics like greater target cell contact, enhanced stability, or stealth behavior—a lower capacity to be recognized by the immune system.

Multifunctional Platforms: By combining therapeutic pharmaceuticals with imaging or diagnostic agents, nano formulations can function as multifunctional platforms. This allows for simultaneous diagnosis and therapy.

Administration Ease: Based on the demands of the patient and the properties of the medicine, nano formulations can be created for a variety of administration routes, such as oral, intravenous, transdermal, or inhalation. This flexibility in drug delivery allows for customized treatment plans. These variables must be carefully taken into consideration, keeping in mind the unique characteristics of the medication and the intended therapeutic objectives, so nano formulations to be applied successfully.

Nano formulation improving drug delivery: The following are some ways that drug distribution can be enhanced by nanoformulations.

Enhancement of Bioavailability: Poorly water-soluble medications can become more soluble thanks to nanoformulations, which increases their absorption and bioavailability. Better absorption of medications is made possible by the protective action of nanoparticles against gastrointestinal tract degradation.

Targeted Administration of Medicines: Targeting particularly tissues, cells, or organs with functionalized nanoparticles can minimize off-target effects and enhance therapeutic results. Adding ligands to targets is known as active targeting. Attaching ligands to the nanoparticles that enable them to identify and bind to certain receptors on target cells is known as active targeting.

Prolonged Release: Drugs can be released from nanoformulations in a regulated or sustained manner, resulting in a longer duration of action and fewer dosage adjustments.

Defence of Pharmaceutical Molecules: Drugs can be more stable in biological settings by using nanoparticles to shield them from enzymatic or chemical processes that could break them down.

Delivery Within Cells: Drugs can be delivered intracellularly more easily with the help of nanoparticles, reaching their intended locations inside cells.

Diminished Adverse Reactions: By limiting the amount of time, medication is exposed to healthy tissues, targeted delivery can lower the likelihood of adverse consequences.

Combination Counselling: Co-delivery of several medications is made possible by nanoformulations, which enables combination therapy with beneficial effects.

Diagnostic Imaging: Nanoparticles can be employed as diagnostic instruments for illnesses or as imaging agents to see how drugs are distributed throughout the body.
Personalized Health Care: Personalized medicine can be advanced by customizing nanoformulations to each patient's unique set of traits.

Non-intrusive Administration Routes: As an alternative to more conventional delivery methods like oral or intravenous injection, nanoparticles can be engineered for non-invasive routes like transdermal or nasal distribution.

Cells/Tissues helping in drug delivery: Different cells and tissues can be used or targeted in medication delivery to improve the safety, effectiveness, and selectivity of medicinal medicines. The following tissues and cells are frequently used in medication delivery.
The inner surface of blood arteries is lined with endothelial cells. It is possible to create nanoparticles to get through the endothelium barrier and deliver them specifically to particularly tissues or organs.

Macrophages: As a component of the immune system, macrophages can be used to carry drugs, particularly to inflammatory regions. It is possible to engineer nanoparticles such that they are specifically delivered to areas of infection or inflammation and are absorbed by macrophages.

Hepatocytes, or liver cells: Because the liver is involved in drug processing, it is frequently the target of drug delivery methods. It is possible to engineer nanoparticles so that they gather in hepatocytes, which would improve the administration of medications that the liver must metabolize or be used to treat liver illnesses.

Cancer Cells: One of the main goals of medication distribution is to target cancer cells. In order to minimize side effects, nanoparticles can be functionalized to recognize and deliver medications to cancer cells only, sparing healthy cells.

Immune cells: Vaccines and immunotherapies can be developed specifically targeting immune cells, such as dendritic cells. Antigens or therapeutic substances that elicit an immune response can be delivered using nanoparticles.

Central Nervous System (CNS) Cells: The blood-brain barrier makes it difficult to transfer drugs to the brain. It is possible to create nanoparticles that can get through this barrier, making the treatment of neurological conditions easier.

Skin Cells: Transdermal drug administration delivers medications locally or systemically by targeting the skin. Drugs can be progressively released from nanoparticles by making them able to permeate the layers of skin.

Bone Cells: Osteoporosis and bone cancer can be treated by targeted medicine delivery using nanoparticles to the bone tissue.

Mucosal Cells: Local medication administration or systemic absorption can be directed towards mucosal surfaces, such as those found in the respiratory and gastrointestinal systems. Through mucosal barriers, medication absorption can be improved by nanoparticles.

Tumor Vasculature: Drug delivery strategies can target the distinct features of blood arteries found within tumors. It is possible to engineer nanoparticles so that they selectively collect in tumor blood arteries, enhancing medication delivery to the tumor.

Red Blood Cells: To improve distribution to particularly organs and extend circulation periods, drug-loaded nanoparticles can be encapsulated or adhered to red blood cells.

Lymphatic System: Drug delivery to lymph nodes and tissues connected to the immune response is made possible by the ability of nanoparticles to specifically target the lymphatic system.

Synovial Cells: To administer anti-inflammatory medications to synovial cells in the joints, such as in rheumatoid arthritis, nanoparticles can be specifically targeted to these cells. Through the utilization of distinct cell and tissue properties, scientists can create drug delivery systems that optimize therapeutic effects while reducing side effects. In order, enhance patient care, the discipline of nanomedicine is still investigating novel strategies for targeted drug delivery.

CONCLUSION

This review makes it clear that the use of nontechnology in medicine and drug delivery has created new avenues for individualized and secure treatment options. In the end, researchers are able to administer medications for longer periods of time with less frequent doses (sustained release), higher precision, and penetration in difficult-to-access tissues through the alteration of molecular size and surface features. There are many benefits to using micro and nanoparticles in biomedicine, particularly when it comes to drug delivery, over traditional methods which include

improved drug delivery, high-performance properties of the product, using less costly drug concentrations in the delivery systems, extending the drug's bioactivity by shielding it from environmental effects in biological media, and more effective treatment with fewer side effects.

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On revealing the Hidden Richness of Fish Diversity from Melghat Region of Maharashtra, India Using DNA Barcoding : A First Approach

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ABSTRACT

The increasing loss of biodiversity globally has led to numerous proposals to intensify efforts to produce a census of all biological diversity and to modernize taxonomy. Over the years, a steady decline has been observed in the abundance and diversity of native fishes in the rivers due to anthropogenic disturbances. The present study was carried out on fish diversity from the major rivers and their tributaries in the Amravati district including the Melghat landscape in Maharashtra. The study was conducted from December 2022 to May 2024. Muscle and fin tissue was collected onsite by following standard protocols to avoid contamination. In this study, a total of 46 species belonging to 36 genera, 16 families, were DNA barcoded using the mitochondrial cytochrome c oxidase subunit I (COI) gene. All of the fish species were discriminated by their COI sequences, showing deep genetic divergence, and were highlighted for further taxonomic investigation. Average Kimura 2-parameter genetic distances within species of families like Channidae, Cyprinidae, and other families 0.75%, 0.82%, and 0.97% are respectively. These values show that COI divergence increases as taxa become less exclusive. Devario aequipinnatus from the family Cyprinidae showed the highest overall GC content at 40.00% , Oreochromis mossambius from the family Chhichlidae had the lowest 28.57% indicating the divergence in the nucleotide composition of fishes. All of the COI sequences obtained were grouped according to their species designation in the maximum likelihood tree that was constructed using MEGA 11 software. This study demonstrated that DNA barcoding has great potential as a tool for fast and accurate species identification and also for highlighting species that warrant further taxonomic investigation.

KEY WORDS: COI, DNA BARCODING, FRESHWATER FISHES, TAPI, WARDHA, PURNA, AMRAVATI DISTRICT, MELGHAT.

INTRODUCTION

Fish account for approximately half of all vertebrates with 34,300 species identified worldwide. Approximately 7.7% of the world's fish are in India, with 994 species classified as freshwater and 1,673 as marine. Sustainable management of genetic resources requires an awareness of fish species taxonomy and systematics. At present many species have become extinct to Indian origin, there is an urgent need to develop a tool to describe all the earth's species so that the associated societal and economic benefits can be derived in addition to evolving strategies for protecting fishes and conserving the resources they constitute). When differentiating between cryptic species of adult fish and larval fish, morphology is not as effective as DNA barcoding, (Krishna et.al. 2012 Shelake etal 2021).

However, DNA barcoding has the potential to identify specific species. The research area's cryptic species, species composition, and several unclear species may all be quickly surveyed using the DNA barcode technology, which can also be used to identify physically similar species (Ko et al. 2013). Applications for barcoding have a great deal of potential appeal in the fishing industry. The authentication of species in fisheries is becoming increasingly dependent on genetics (Ardura et al 2013). DNA barcoding, which is supported by Hebert et al. (2003a, 2003b), aims to make it easier to recognize the growing number of taxa that are unfamiliar in biological conservation and biodiversity surveys. The 652 base pair target DNA fragment regarding fishes is highly suggested to be located close to the 5′ ends of the mitochondrial cytochrome oxidase subunit I gene (Zhang et.al. 2011).

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DNA barcoding's main objectives are to create reference libraries of barcode sequences for recognized species so that trustworthy molecular tools for identifying species in the wild can be created (Hubert et. al. 2008). Numerous marine and freshwater fish have benefited from the technology's application. In addition to successfully classifying unknown specimens into recognized species, DNA barcoding is an emerging method for species identification that can also identify genetically distant populations. The positive outcomes have spurred global initiatives to expedite the process of identifying cryptic species and standardize the screening of species diversity (Lakra et al. 2016). Furthermore, intraspecific genetic variation in fish species can be discovered using the DNA barcoding method (Decru et al.2016).

96 species under 52 genera and 19 families from Melghat Tiger Reserve, 17 species from the Salbardi region near Morshi taluka of Amravati district, and 36 species belonging to 11 families from the rivers of the Amravati district respectively (Yadav 2005; Wagh et.al 2008; Wankhade 2015) have been reported, but the identification of fishes was done by a classical method which sometimes could give discrepancies in species confirmation. To overcome this limitation, the use of molecular tools has proven beneficial, and the present study is one of the few and first in this region to analyse the fish diversity using DNA barcoding.

MATERIAL AND METHODS

Study Area – Major rivers and their tributaries in the Amravati district : Amravati district lies between – (20° 32' and 21° 46' NL) and (76° 37' and 78° 27' EL). It occupies an area of 12,149.7 sq. Km. This district is situated right in the center of the northern border of Maharashtra State. The district is an undulating plain of black soil of a fertile type, its richest tracts being perhaps in the neighborhood of the Wardha and the Purna rivers. It is watered by several streams which rise in the Satpudas in the north. The climate of this district is characterized by a hot summer and general dryness throughout the year except during the southwest monsoon season. The temperature of the district varies between 12.4°C to 44.5°C and the average rainfall is 841.8.

The district is bestowed with three major rivers namely the Tapi river, Purna river, and the Wardha river, and their important tributaries like the Sapan river, Chandrabhaga river, Pedhi river, Sipna river, Bembla river, Gadga river, Khandu river, Khapra river, Dolar river, were surveyed during the study. Tapi lies towards the southern part of the Melghat hills, The Purna, the largest of them rises near Bhainsdehi in the Betul district of Madhya Pradesh at a height of just over 760 meters in the Satpudas, The Wardha river rises to the east of Multai in Madhya Pradesh and forms the eastern boundary of the Amravati district and receives several short tributaries on its right flowing within the district.

In the Amravati district, the area of Melghat is drained by the Khandu river, the Khapra river, the Sipna river, the Gadga river and the Dolar river which are tributaries of the Tapi river. The climate of Melghat is tropical and the forest is dry and deciduous in which December is the coldest month 13°C and 22°C is the maximum temperature. Annual rainfall amounts to 2250 mm which gradually decreases towards the north where it is recorded to 1000mm only.

Figure 1: Map showing surveyed station in the rivers of the Amravati district including Melghat

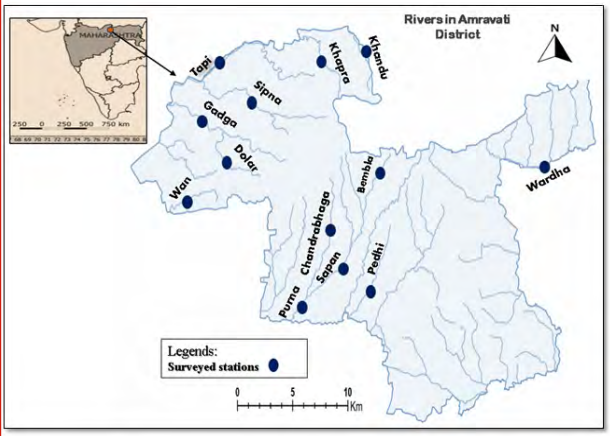


Figure 2: A-Tapi river, B- Upparwardha reservior, C-Purna river, D-Pedhi river, E-Sapan river, F-Chandrabhaga river, G-Bembla river, H-Gadga river, I- Sipna river

View of rivers in the Amravati district including Melghat surveyed during the study period to document fish diversity

Methodology

Sample collection: Fishes were collected from different rivers of the Amravati district including Melghat namely the major rivers like the Tapi, Purna, and Wardha and their major tributaries. All the fishes were identified morphometrically, with the help of Day (1875-78, 1889), Koumans (1953), Talwar and Jhingran (1991) FAO-Fisheries Identification Sheets (1974). Voucher specimens were maintained in the college departmental laboratory. Samples were collected from December 2022 to May- 2024. Digital photographs of all the fishes were taken immediately and the fish were

stored at (-20°C), and approximately 100 mg of muscle tissue from each species was preserved in 70% ethanol until used. Further procedures from the DNA isolation to the Fish identification were performed with the help of the experts in the Bioscience Barcoding Laboratory, Banglore, India.

Table 1. Riverwise stations were covered during the study.

Rivers	Surveyed Stations	GPS coordinates
Tapi	Rangubeli	77.14015°N and 21.71775°E
	Amner fort	76.78457°N and 21.52881°E
Sipna	Semadoh	77.31222°N and 21.497444°E
	Kolkas	77.17418°N and 21.50213°E
	Harisal	77.124218°N and 21.523066°E
Gadga	Amner fort	76.78457°N and 21.52881°E
Dolar	Dhakna	77.05934°N and 21.433778 °E
Wardha	Upparwardha	78.022228°N and 21.30918°E
Purna	Asegaon Purna	77.569182°N and 21.1267°E
Pedhi	Walgaon	77.70328°N and 20.99899°E
Sapan	Sawali	77.485433°N and 21.310439°E
Chandrabhaga	Wadgaon	77.438672°N and 21.279106°E
Bembla	Bhuikhed	78.014728° N and 20.654545°E

Experimental Methods

1. DNA was isolated from the tissue sample of fish provided. Its quality was evaluated on 1.0 % agarose gel, and a single band of high-molecular-weight DNA was observed.
2. Fragment of the COI (cytochrome oxidase-I) gene was amplified by Fish F1 and Fish F2; Fish R1 and Fish R2 primers. A single discrete PCR (Polymerase chain reaction) amplicon band of 700 bp was observed when resolved on agarose gel.

Fish F1- 5'TCAACCAACCACAAAGACATTGGCAC3'
Fish F2- 5'TCGACTAATCATAAAGATATCGGCAC3'
Fish R1- 5'TAGACTTCTGGGTGGCCAAAGAATCA3'
Fish R2- 5'ACTTCAGGGTGACCGAAGAATCAGAA3'

1. The PCR amplicon was purified to remove contaminants.
2. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with Fish F1 and Fish F2; Fish R1 and Fish R2 primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.
3. A consensus sequence of COI genes was generated from forward and reverse sequence data using aligner software.
4. The COI gene sequence was used to carry out BLAST (Basic Local Alignment Search Tool) with the 'nr'(non-redundant) database of the NCBI (National Center for Biotechnology Information) GenBank database. Based on the maximum identity score first ten sequences were selected and aligned using the multiple alignment software program Clustal W.

Data analysis: In total 46 species sequences were aligned using Clustal W and pairwise evolutionary distance was determined by the Kimura 2-parameter method (Kimura 1980) using the software program MEGA 11. Reference sequences of these five species were retrieved from NCBI (National Center for Biotechnology Information) GenBank and the familywise phylogenetic tree was constructed using the maximum likelihood method. To verify the robustness of the internal nodes of the ML tree, bootstrap analysis was carried out using 100 pseudoreplicates. The base composition and genetic distance of each fish species barcoded was obtained using the software MEGA 11.

RESULTS AND DISCUSSION

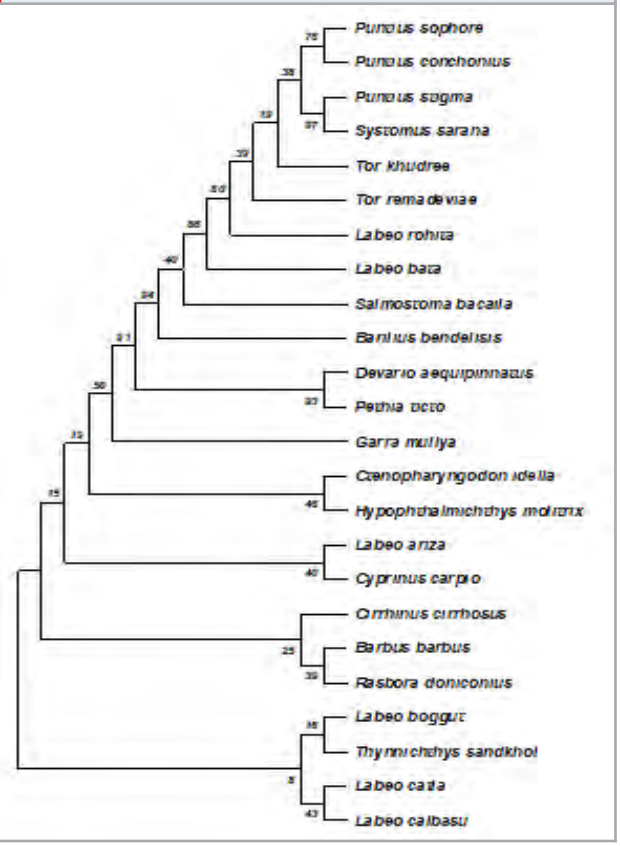
A total of 46 COI of different freshwater fish species were analyzed in the Amravati district including the Melghat landscape from 17 families and 35 genera. Among the 46 species identified 18 species are newly recorded for the Amravati district. The BLAST searches by each sample sequence in GenBank revealed the closest matches with sample 1 depicting the scientific name, IUCN category, and accession number (NCBI). COI barcodes obtained ranged from 604 to 664 bp, with an average of 640 bp (table 2).

Maximum diversity has been seen in the Wardha river with some 17 species of fishes, followed by the Tapi from the Melghat landscape which is 15,the Purna river, the Pedhi river, the Chandrabhaga river,the Sapan river, the Bembla river, the Sipna river from the Melghat.

Nucleotide content analysis showed the following average frequencies: Adenine (A): 22.43%, Thymine (T): 28.21%, Cytosine (C): 25.32%, and Guanine (G): 24.04%. Overall nucleotide content and content at each codon position are presented in Table 3. *Devario aequipinnatus* from family Cyprinidae showed the highest overall GC content at 40.00% (G: 15.71% + C: 24.29%) while *Oreochromis mossambius*

from family Chhichlidae had the lowest at 28.57% (G: 15.71%+ C: 12.86%).. Overall genetic distance within the family Channidae is 0.75%, Cyprinidae 0.82% and the other remaining fishes of different families is 0.97%.

Figure 3: The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved nucleotide sequences of the Cyprinidae family using MEGA11.



The genetic distance between the fishes of different families is highest displayed increasing genetic variation at increasing taxonomic levels. The phylogenetic tree shows that the species are all related, but some are more closely related than others. For example, in the cyprinidae family, the species *Puntius sophore* and *Puntius conchonius* are more closely related to each other than they are to any other species in the tree. This is because they share a more recent common ancestor and are similarly the same in the other two phylogenetic trees. *Clarias batrachus* clusters with *Heteropneustes fossilis* with high support (92%). This suggests that these species share a more recent common ancestor than with other species in the tree. They belong to the family Clariidae, which are air-sac catfish.

The clade with *Oreochromis mossambicus* (Oreochromidae) and *Pygocentrus nattereri* (Serrasalmidae) has moderate support (70%). This grouping is less certain than the Clarias-Heteropneustes grouping. It suggests these lineages may have diverged from a common ancestor more distantly than the Clarias and Heteropneustes species. *Anguilla bengalensis* (Anguillidae) and *Schistura savona* (Nemacheilidae) form a separate clade with high support (76%). This means they likely share a more recent common ancestor than with other species on the tree. Several well-supported clades are present at the bottom of the tree. These include the clade with *Pangasius pangasius* and Mystus bleekeri (both Pangasiidae) and the clade with *Xenentodon cancila* (Notopteridae) and *Notopterus notopterus* (Notopteridae).

This suggests strong evolutionary relationships within these families. DNA barcoding can be used for the authentication of documenting the fish diversity within the area of high potential for biodiversity existence due to the supporting ecology present there.

The primers used in the study were able to target and amplify the COI gene region in all 46 specimens of fish. No insertions and deletions were found in the sequence and upon translation, no stop codons were detected. This supports the hypothesis that mitochondrial COI sequence can be used as a standard region for identifying animal species (Hajibabaei et al. 2007b). Fishes like *Tor khudree* and *Tor remadevii* which are recorded from the Gadga river and Tapi river of the Melghat landscape respectively are highly significant fishes ecologically and demanded by locals due to their taste falling under the Critically Endangered and Vulnerable category of IUCN red-list had been identified at the molecular level for the first time in the Amravati district which will help the respective management authority to take actions for their conservations.

In terms of average genetic distances within various taxonomic levels, an increasing pattern was observed as the taxa became less exclusive. In which *Systomus sarana* was recorded for the first time in the Melghat landscape. 18 fishes shown with asterisk marks are newly reported for the Amravati district as compared to the previous record done by (Wankhade, 2015) which too based on molecular evidence (table 2). several species like *Barbus barbus, Barilius bendelisis, Cirrhinus cirrhosus, Devario aequipinnatus* make new records for the Amravati district. Some species like *Barilius bendelisis, Rasbora doniconius, Thynnichthys sandkhol, Devario aequipinnatus, Tor remadevii* (VU), *Anguilla bengalensis* (NT), *Tor khudree*(CR) come under the red-list of IUCN category.

All the fishes mentioned in (Table 2) are barcoded and this has been the first attempt for the fish fauna in the Amravati district including Melghat. Earlier the work done by (Yadav 2005; Wagh et.al., 2008; Wankhade, 2015) was only based on the classical level. Table 3 provides the nucleotide composition which infers that there is variation in the nucleotide composition and hence gives evidence that fishes are different from one another at the genetic level. The decline in the number of native fish species in the Amravati district including the Melghat landscape and the changing species composition over time due to the changes in the ecological factors in the rivers call for strict enforcement of regulatory measures that will protect the native fish species found in the rivers.

Table 2- Fishes with their accession number in the NCBI database

FAMILY	SCIENTIFIC NAME	IUCN	Accession Number
Angullidae (2)	*Anguilla bengalensis* (Mottled eel)	(NT)	MK572031.1
	Macrognathus pancalus (Spiny eel)	(LC)	JX983358.1
Cyprinidae (24)	*Ariza labeo* (Labeo)	(DD)	FJ459477.1
	*Barbus barbus**(The common barbel)	(LC)	ON097307.1
	*Barilius bendelisis**(Indian hill trout)	(VU)	MK277203.1
	*Cirrhinus cirrhosus**(Mrigal carp)	(LC)	MK572126.1
	Ctenopharyngodon idella (Grass carp)	(LC)	OP575587.1
	Cyprinus carpio (Common carp)	(LC)	JX983284.1
	*Devario aequipinnatus** (Giant danio)	(VU)	MK599491.1
	Garra mullya (Suckerfish)	(LC)	JX983296.1
	*Hypophthalmichthys molitrix** (Silver carp)	(LC)	JX983319.1
	Labeo boggut (Minor carp)	(LC)	JX983331.1
	Labeo catla (Catla)	(LC)	JX983340.1
	Labeo calbasu (Labeo)	(DD)	JX983340.1
	Labeo rohita (Rohu)	(LC)	JX983352.1
	Labeo bata (Bata)	(LC)	MH156965.1
	Pethia ticto (Ticto barb)	(LC)	MF966244.1
	Puntius sophore (Stigma barb)	(LC)	MK599535.1
	Puntius stigma (Pool barb)	(LC)	JX260943.1
	*Puntius conchonius** (Rosy barb)	(LC)	JN965201.1
	Rasbora doniconius (Blackline rasbora)	(VU)	MN342807.1
	*Systomus sarana**(Olive barb)	(NT)	JX983460.1
	Salmostoma bacaila (Large razorbelly minnow)	(LC)	EU417789.1
	*Thynnichthys sandkhol** (Sandkhol)	(VU)	JX260985.1
	*Tor khudree**(Blue–finned mahaseer)	(CR)	KX946824.1
	*Tor remadevii**(Orange– finned mahaseer)	(VU)	MG769040.1
Channidae (4)	*Channa punctatus* (Spotted snakehead)	(LC)	MN178288.1
	*Channa marulius** (Bullseye snakehead)	(DD)	OL638201.1
	Channa striata (Striped snakehead)	(LC)	OP575576.1
	*Channa gachua**(Dwarf snakehead)	(LC)	MK599523.1
Bagridae (2)	*Mystus bleekeri**(Day's mystus)	(DD)	OP661359.1
	Mystus cavasius (Gangetic mystus)	(LC)	MK577973.1
Siluridae (2)	*Wallago attu* (Wallago attu)	(VU)	MK577971.1
	Ompok bimaculatus (Butter catfish)	(DD)	OM273996.1
Ambassidae (1)	*Parambassis ranga* (Indian glassy fish)	(LC)	KY694517.1
Belonidae (1)	*Xenentodon cancila* (Freshwater garfish)	(CR)	MK359936.1
Cichlidae (1)	*Oreochromis mossambius** (Mazambique tilapia)	(LC)	KU565826.1
Clarridae (1)	*Clarius batratus* (Walking catfish)	(LC)	MG988401.1
Cobitidae (1)	*Lepidocephalichthys guntea** (Guntea loach)	(LC)	MH197211.1
Gobiidae (1)	*Glossogobius giuris* (Tank gobby)	(LC)	MN172285.1
Heteropneustidae (1)	*Heteropneustes Fossilis* (Fossil cat)	(LC)	MK572259.1
Mastacembelidae(1)	*Mastacembelus armatus* (Zig–zag eel)	(LC)	JX983365.1
Nemacheilidae (1)	*Schistura savona** (Stone loach)	(VU)	KJ542585.1
Notopteridae (1)	*Notopterus notopterus* (Bronze featherback)	(NT)	MK336899.1
Pangassidae (1)	*Pangasius pangasius** (Pangas)	(VU)	MK572424.1
Serrasalmidae (1)	*Pygocentrus nattereri** (Red –bellied piranha)	(LC)	MG752582.1

Table 3. Nucleotide composition of fish barcoded

Species	T	C	A	G	Total
Labeo ariza	25.71428571	27.1428571	21.428571	25.7142857	70
Barbus barbus	31.42857143	24.2857143	21.428571	22.8571429	70
Barilius bendelisis	25.71428571	27.1428571	17.142857	30	70
Cirrhinus cirrhosus	31.42857143	24.2857143	18.571429	25.7142857	70
Cyprinus carpio	22.85714286	30	20	27.1428571	70
Devario aequipinnatus	48.57142857	14.2857143	21.428571	15.7142857	70
Garra mullya	32.85714286	24.2857143	17.142857	25.7142857	70
Hypophthalmichthys molitrix	30.43478261	24.6376812	21.73913	23.1884058	69
Labeo catla	28.57142857	27.1428571	20	24.2857143	70
Labeo calbasu	31.42857143	24.2857143	20	24.2857143	70
Labeo rohita	31.42857143	22.8571429	21.428571	24.2857143	70
Labeo bata	20	31.4285714	22.857143	25.7142857	70
Puntius sophore	17.14285714	32.8571429	25.714286	24.2857143	70
Puntius arenatus	30	21.4285714	25.714286	22.8571429	70
Puntius conchonius	25.71428571	25.7142857	27.142857	21.4285714	70
Rasbora rasbora	37.14285714	25.7142857	15.714286	21.4285714	70
Systomus sarana	27.14285714	25.7142857	22.857143	24.2857143	70
Salmostoma bacaila	21.42857143	31.4285714	21.428571	25.7142857	70
Thynnichthys sandkhol	34.28571429	22.8571429	15.714286	27.1428571	70
Tor khudree	27.14285714	25.7142857	27.142857	20	70
Tor remadeviae	24.28571429	31.4285714	21.428571	22.8571429	70
Channa punctata	24.28571429	25.7142857	27.142857	22.8571429	70
Channa marulius	34.28571429	21.4285714	12.857143	31.4285714	70
Channa striata	24.28571429	35.7142857	24.285714	15.7142857	70
Channa gachua	30	27.1428571	20	22.8571429	70
Mystus bleekeri	25.71428571	25.7142857	25.714286	22.8571429	70
Mystus cavasius	27.14285714	24.2857143	22.857143	25.7142857	70
Wallago attu	22.85714286	30	24.285714	22.8571429	70
Ompok bimaculatus	25.71428571	27.1428571	24.285714	22.8571429	70
Parambassis ranga	27.14285714	27.1428571	18.571429	27.1428571	70
Xenentodon cancila	28.57142857	15.7142857	22.857143	32.8571429	70
Oreochromis mossambius	40	12.8571429	31.428571	15.7142857	70
Clarias batratuss	20	27.1428571	25.714286	27.1428571	70
Glossogobius giuriss	28.57142857	24.2857143	18.571429	28.5714286	70
Heteropneustes fossilis	30	21.4285714	25.714286	22.8571429	70
Mastacembelus armatus	17.14285714	30	27.142857	25.7142857	70
Schistura savona	31.42857143	24.2857143	20	24.2857143	70
Notopterus notopterus	22.85714286	22.8571429	24.285714	30	70
Pangasius pangasius	30	22.8571429	18.571429	28.5714286	70
Pygocentrus nattereri	25.71428571	28.5714286	27.142857	18.5714286	70
Lepidocephalichthys guntea	32.85714286	24.2857143	18.571429	24.2857143	70
Ctenopharyngodon idella	28.6	27.1	20.0	24.3	70
Labeo boggut	28.6	25.7	18.6	27.1	70
Pethia ticto	18.6	27.1	22.9	31.4	70
Anguilla bengalensis	27.1	24.3	22.9	25.7	70
Macrognathus pancalus	25.7	25.7	28.6	20.0	70

A - Adenine. G -Guanine, T-Thymine, C-Cytosine

Figure 4: The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved nucleotide sequences of the Channidae family using MEGA 11.

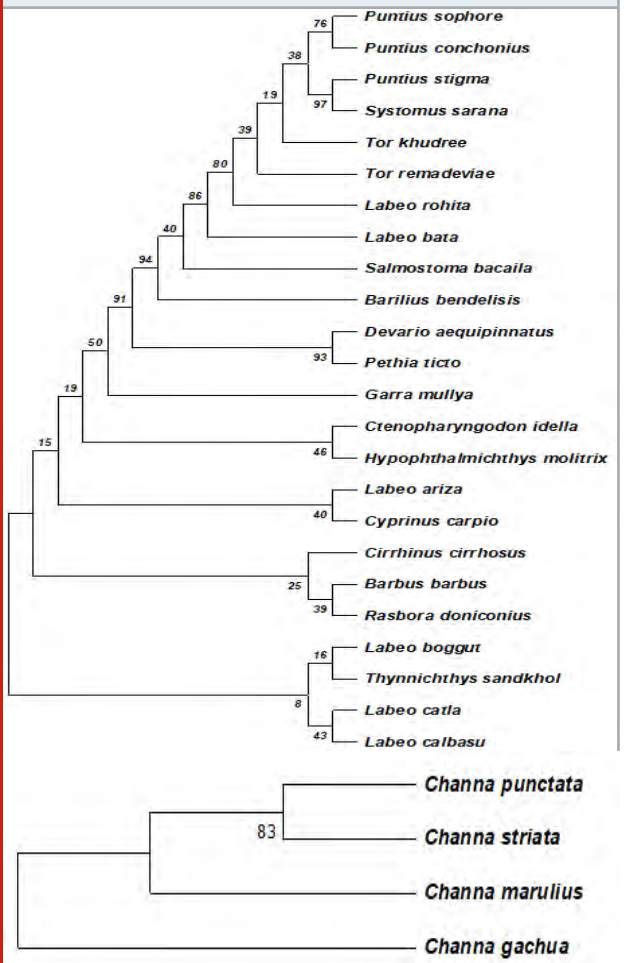
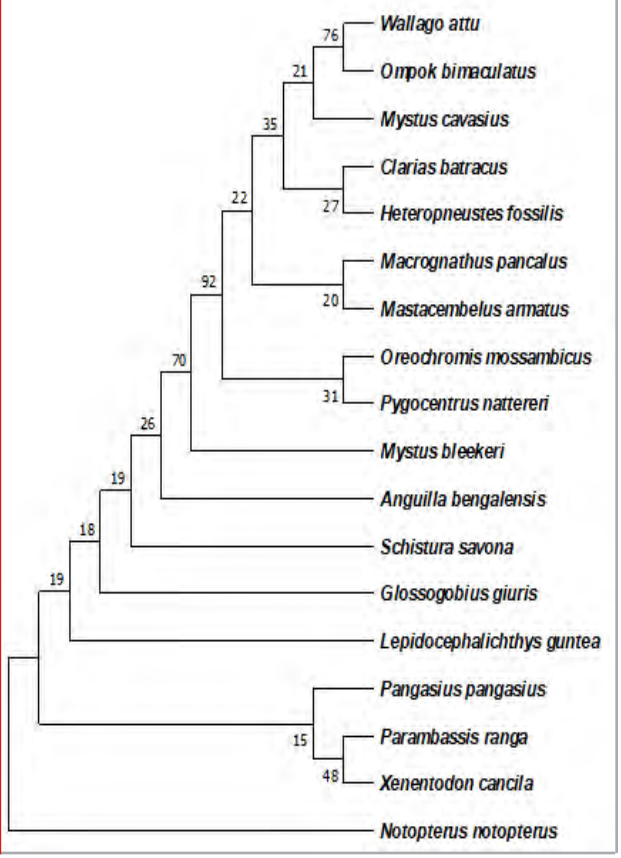


Figure 5: The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved nucleotide sequences of different families using MEGA 11.



CONCLUSION

In this study, DNA barcoding using the mitochondrial COI gene was successful in discriminating 46 species of fish in the Amravati district including the Melghat region. Furthermore, DNA barcoding has advanced the study of the river's ichthyofauna by providing new taxonomic information at the molecular level as well as identifying previously unreported species. Effective management of the Amravati district native fish populations, which have been in decline both in abundance and diversity due to anthropogenic disturbances and increasing use of the rivers for fisheries. Finally, the COI sequences submitted to BOLD and GenBank can aid others in accurate species identification once these are made publicly available. Additional specimens of those species with very few vouchers should be collected further to assess better the uniqueness of the barcode sequence of each species.

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Pharmacokinetic and ADMET Properties of Bio Actives from *Catharanthus roseus* and its Associated Molecular Docking Against Thioredoxin-Interacting Protein and Protein Tyrosine Phosphatase 1B for Management of Type 2 Diabetes

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ABSTRACT

Catharanthus roseus Linn., often known as Shadabahar, is a medicinal herb whose leaf extract has traditionally been used to prevent type 2 diabetes. The study's objectives were to identify lead small molecules as diabetic medications phytocompounds using molecular docking, as well as to determine the bioactive pharmacokinetics of plants and ADMET (absorption–distribution–metabolism–excretion–toxicity) characteristics. In silico analysis of ADMET properties of four phytocompounds and a common synthetic medicine using PkCSM software, as well as receptor-ligand binding energy along with interaction studies through molecular docking for phytocompounds found in *Catharanthus roseus* Linn. on tyrosine phosphatase 1B or TP1B (PDB ID: 2BGD) followed by Thioredoxin interacting protein or TXNIP (PDB ID: 4LL1) as a causative agent for T2D was done. The molecular docking was carried out using AutoDock 4.2 to determine the optimal binding affinity & energy. The molecular interaction was visualised using the molecular graphics laboratory (MGL)/ Chimaera X tool. Petunidin, Hirsutidin, Catharanthine, and Vindoline from *Catharanthus roseus* & Rosiglitazone demonstrated satisfactory findings on the numerous parameters used to evaluate the ADMET qualities.The molecular docking revealed that Hirsutidin had a lower binding energy (-7.62 Kcal/mol) on the TP1B receptor than Catharanthine on the TXNIP receptor (-5.8 Kcal/mol) when compared with synthetic medicines Rosiglitazone which had binding energy (-7.12 Kcal/mol) & (- 4.75 Kcal/mol) on the TP1B and TXNIP receptors. Finally, the predictions indicated that Hirsutidin or Catharanthine might represent a promising lead option for T2D prevention. It is proposed that the current prediction be validated with experimental toxicology & pharmacological assays in the future.

KEY WORDS: CATHARANTHUS ROSEUS; ADMET PROPERTIES; MOLECULAR DOCKING; TYROSINE PHOSPHATASE 1B; THIOREDOXIN INTERACTING PROTEIN.

INTRODUCTION

The term "diabetes mellitus" refers to a collection of metabolic diseases characterised by elevated blood glucose levels brought on by deficiencies in either the secretion or the function of insulin (Ganie and Kotwal, 2012). Based on its pathophysiology, diabetes mellitus can be divided into three groups (IDF , 2013). An auto-immune response is the cause of type 1 diabetes (Liu ,et al.; 2017). Another subtype found in pregnant women is gestational diabetes mellitus. Insulin sensitivity and declining beta cell insulin production are the root causes of type 2 diabetes (Yoshihara , et al.; 2014). Numerous recorded pieces of evidence support the idea that pancreatic beta cell loss plays a part in the onset of diabetes, (Alhawiti ,et.al;2017 Sanyaolu et al 2023).

Thioredoxin-interacting protein, or TXNIP for short, is a protein that regulates metabolism and is present in key organs such as the liver, skeletal muscles, and adipose tissues. TXNIP are proteins that belong to the alpha arrestin family. TXNIP interacts with Thioredoxins (TXN1 and TXN2), causing them to become less active. Through controlling adipogenesis, peripheral glucose absorption, beta cell activity, and other processes, TXNIP is crucial for the regulation of glucose and lipid metabolism. Insulin sensitivity is decreased and pancreatic beta cell death is caused by overexpression of TXNIP (Alhawiti, et al; 2017).

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Table 1. PubChem id , Molecular Formula and Smiles of the bio actives of *Catharanthus roseus* & Rosiglitazone

Compound	PubChem id	Molecular Formula	Smiles
Petunidin	441774	$C_{16}H_{13}O_7$+	COC1=CC(=CC(=C1O)O) C2=[O+]C3=CC(=CC(=C3C=C2O)O)O
Hirsutidin	441694	$C_{18}H_{17}O_7$+	COC1=CC(=C2C=C (C(=[O+]C2=C1)C3=CC(=C (C(=C3)OC)O)OC)O)O
Catharanthine	5458190	$C_{21}H_{24}N_2O_2$	CCC1=CC2CC3 (C1N(C2)CCC4=C3NC5= CC=CC=C45)C(=O)OC
Vindoline	260535	$C_{25}H_{32}N2O_6$	CCC12C=CCN3C1C4(CC3) C(C(C2OC(=O)C)(C(=O) OC)O)N(C5=C4C=CC(=C5)OC)C
Rosiglitazone	77999	$C_{18}H_{19}N3O_3S$	CN(CCOC1=CC=C(C=C1) CC2C(=O)NC(=O) S2)C3=CC=CC=N3

Figure 1: Molecular formula and 2D Structure of the bio actives of *Catharanthus roseus* & Rosiglitazone

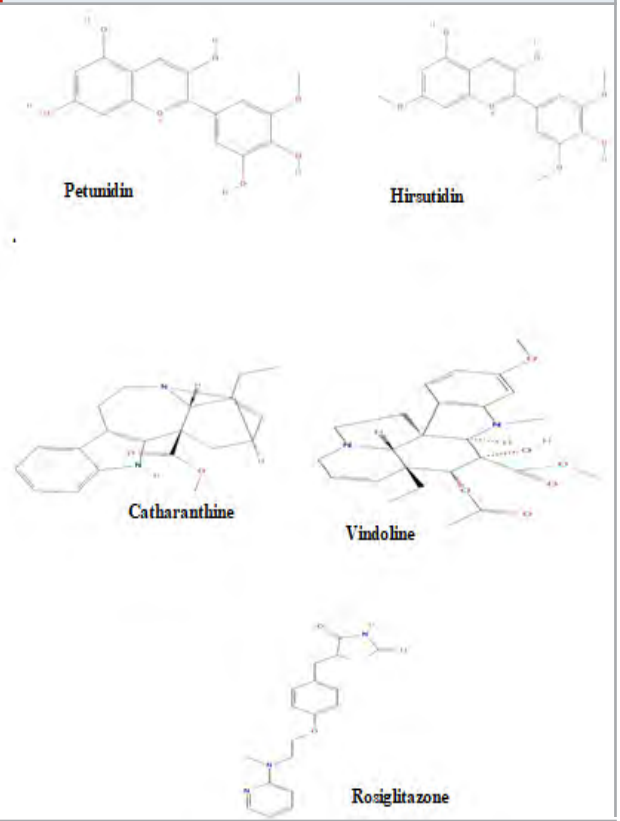


Figure 2: The ribbon shaped 3D structure of the Thioredoxin interacting protein & Tyrosine phosphatase

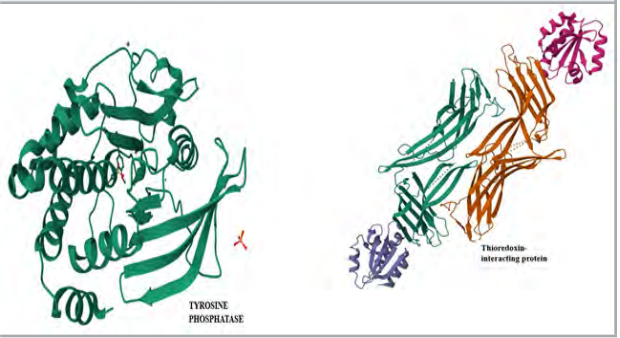
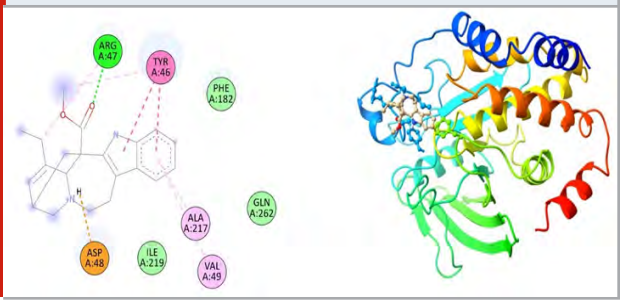


Figure 3: The docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Catharanthine calculated by molecular docking study.



The TXNIP protein binds to TXN1, interfering with TXN1's capacity to decrease oxidised protein, leading to oxidative stress and increased apoptotic possibilities. After migrating to the mitochondria, TXNIP competes with apoptosis signal regulating kinase 1 (ASK-1) to interact with TXN2, causing ASK-1 to be released. ASK-1 frequently binds to TXN2, inhibiting its activity (Yoshihara , et al.; 2014). Pancreatic beta cells' apoptotic signalling cascade is triggered when the released ASK-1 is phosphorylated and activated as a result of TXNIP activity. Diabetes is primarily caused by beta cell dysfunction and reduced insulin production (Wondafrash , et al; 2020 Sanyaolu et al 2023).

Table 2. Molecular properties of the bio actives of *Catharanthus roseus* & Rosiglitazone

PROPERTY	MOLECULAR PROPERTIES				
	Petunidin	Hirsutidin	Catharanthine	Vindoline	Rosiglitazone
Molecular Weight	317.273	345.327	336.435	456.539	357.435
LogP	2.9175	3.5235	3.1753	1.6413	2.4909
#Rotatable Bonds	2	4	2	4	7
#Acceptors	6	6	3	8	6
#Donors	5	3	1	1	1
Surface Area	129.425	142.793	147.394	193.630	150.126

Figure 4: The docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Hirsutidin calculated by molecular docking study.

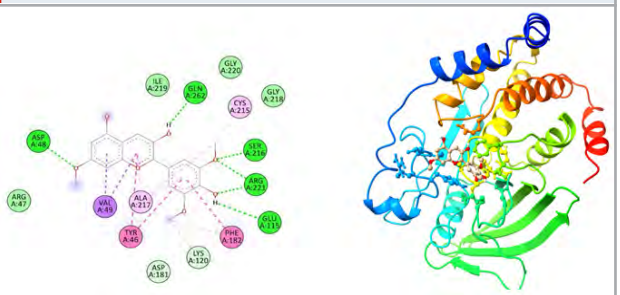


Figure 5: The docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Petunidin calculated by molecular docking study.

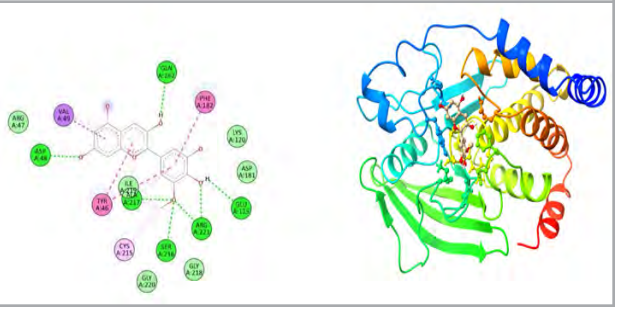


Figure 6: The docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Vindoline calculated by molecular docking study.

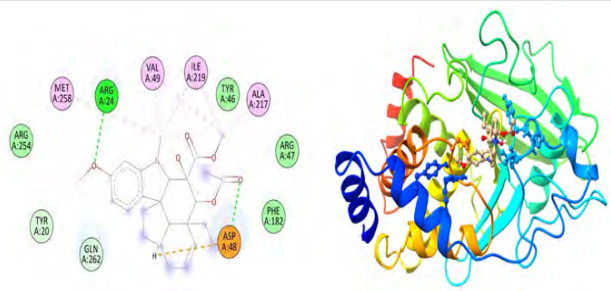
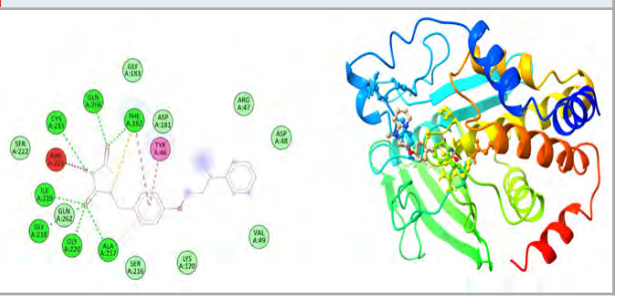


Figure 7: The docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Rosiglitazone calculated by molecular docking study.



A non-transmembrane enzyme called protein-tyrosine phosphatase 1B (PTP1B) is present on the endoplasmic reticulum (ER). Its importance comes from the fact that it negatively regulates both insulin and leptin signalling. Insulin receptor substrate proteins, also known as PTP1B, are the main substrates of the insulin receptor (IR) and are dephosporylated, or to put it another way, free of a phosphate group. PTP1B in leptin removes phosphate from JAK2, a tyrosine kinase known as Janus kinase 2.

It has lately been discovered to be an important factor in the development of tumours and has been connected more directly to breast cancer. It is also considered a possible pharmacological target since its blockage may prevent type 2 diabetes, obesity, and some types of cancer. Because of its highly conserved positively charged active site pocket, protein tyrosine phosphatase 1B (PTP1B) is a useful target for the treatment of type 2 diabetes and obesity.

However, PTP1B is difficult to target for drug discovery. Significant progress has been achieved in the creation of highly potent and specific PTP1B inhibitors that bind to both the active and catalytic sites. To enhance the pharmacological characteristics of PTP1B inhibitors, a number of approaches are being investigated (Rohan et al 2011). In addition to being a therapeutic target for the treatment of insulin resistant conditions including obesity and type 2 diabetes mellitus, protein tyrosine phosphatase 1B (PTB1B) is becoming more and more important in the pathogenesis of the resistance to insulin in diabetes mellitus (Radhika et al,2012). Moreover, PTP1B contributes to the suppression of leptin and insulin signalling. Consequently,

Table 3. ADMET properties of the bio actives of *Catharanthus roseus* & Rosiglitazone

PROPERTY	Petunidin	Hirsutidin	Catharanthine	Vindoline	Rosiglitazone
			ABSORPTION		
Water solubility	-2.943	-3.583	-3.399	-3.414	-3.762
Caco2 permeability	-0.47	0.041	1.114	0.222	0.964
Intestinal absorption (human)	84.429	84.297	93.597	96.576	93.757
Skin Permeability	-2.735	-2.735	-2.932	-3.088	-2.844
P-glycoprotein substrate	Yes	Yes	Yes	Yes	No
P-glycoprotein I inhibitor	No	No	Yes	Yes	Yes
P-glycoprotein II inhibitor	No	Yes	No	Yes	No
			DISTRIBUTION		
VDss (human)	0.837	-0.215	1.485	0.542	-0.183
Fraction unbound (human)	0.182	0.054	0.312	0.265	0.078
BBB permeability	-1.415	-1.292	0.287	-0.261	-0.727
CNS permeability	-3.48	-3.018	-1.939	-3.375	-2.785
			METABOLISM		
CYP2D6 substrate	No	No	Yes	No	No
CYP3A4 substrate	No	Yes	Yes	Yes	Yes
CYP1A2 inhibitior	Yes	Yes	No	No	No
CYP2C19 inhibitior	No	Yes	No	No	Yes
CYP2C9 inhibitior	No	Yes	No	No	No
CYP2D6 inhibitior	No	No	Yes	No	No
CYP3A4 inhibitior	No	No	No	No	No
			EXCRETION		
Total Clearance	0.647	0.746	1.167	0.511	0.107
Renal OCT2 substrate	No	No	Yes	No	No
			TOXICITY		
AMES toxicity	No	No	Yes	No	No
Max. tolerated dose (human)	0.534	0.657	-0.572	-0.661	0.066
hERG I inhibitor	No	No	No	No	No
hERG II inhibitor	No	No	Yes	Yes	No
Oral Rat Acute Toxicity (LD50)	2.459	2.32	3.139	3.225	2.692
Oral Rat Chronic Toxicity (LOAEL)	2.45	1.68	1.488	1.599	1.415
Hepatotoxicity	No	No	No	No	Yes
Skin Sensitisation	No	No	No	No	No
T.Pyriformis toxicity	0.294	0.341	0.445	0.295	1.038
Minnow toxicity	2.463	1.626	-0.727	1.365	1.63

PTP1B inhibitors offer therapeutic potential for the treatment of Type II diabetes as well as obesity. There is persuasive evidence that small molecule PTP1B inhibitors may be helpful in managing insulin resistance early on, resulting in a T2DM and obesity preventive strategy (Rao et al 2006).

According to Ayurvedic research, the flower of *Catharanthus roseus* is said to treat diabetes. Several laboratory investigations have shown that *Catharanthus roseus* extracts derived from various plant components, such as the root, leaf, flower, and stem, have hypoglycemic action. Throughout India, people have been using the ancient medicinal plant *Catharanthus roseus* to cure diabetes. Patients with diabetes mellitus are given various plant components, such as leaves, flowers, and stems (Jayanthi et al 2010). Flowers of *Catharanthus roseus* include numerous bioactives such as Petunidin, Hirsutidin, Catharanthine, and Vindoline. Bioactives are a category of natural compounds with varying polyphenolic structures that have anti-oxidant, anti-diabetic, and anti-aging properties (Saul et al 2009).

Table 4. Binding energy (kcal/mol) and inhibition constant (μM) values of the docking complexes of proteins with ligands calculated by molecular docking study.

Protein	Petunidin		Hirsutidin		Catharanthine		Vindoline		Rosiglitazone	
	(kcal/mol)	μM	(kcal/mol)	μM	(kcal/mol)	μM	(kcal/mol)	μM	(kcal/mol)	μM
TXNIP (4LL1)	-4.04	1100	-4.67	378.3	-5.8	56.12	-3.51	2650	-4.75	327.53
PTP1B(2BGD)	-6.81	10.11	-7.62	2.61	-5.73	63.18	-4.79	308.1	-7.12	6.02

Figure 8: The docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Catharanthine calculated by molecular docking study.

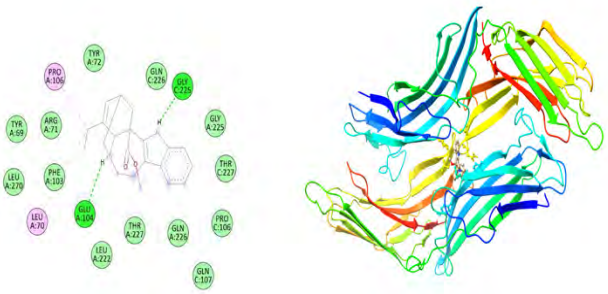


Figure 9: The docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Hirsutidin calculated by molecular docking study.

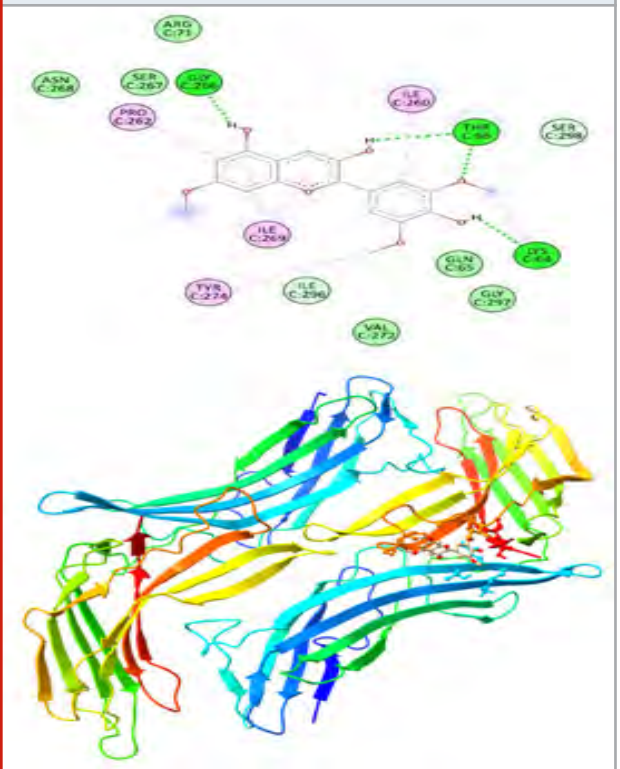
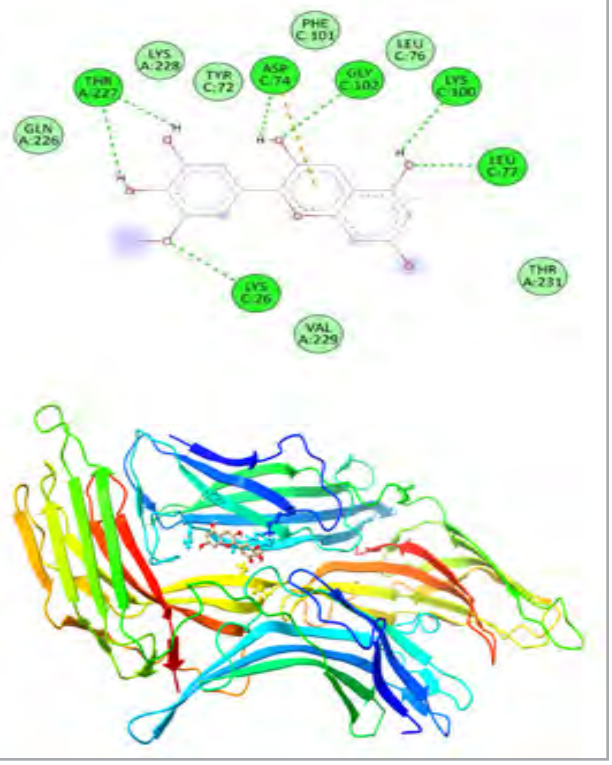


Figure 10: The docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Petunidin calculated by molecular docking study.



Other phenolic compounds, including petunidin, are naturally occurring polyphenols that preserve pancreatic beta cells, promote their growth, lower beta cell death, and reduce oxidative stress, all of which aid in the prevention and management of Type 2 Diabetes Mellitus (Sun, et al; 2020). Identifying targets and predicting innovative medications In-silico approaches have been quite important (Wadood et al., 2013). With the use of bioinformatics tools and AutoDock Tools to assess the docking score, the current work examined the bioactive from *Catharanthus roseus* inhibitory effect on TXNIP and PTP 1B protein.

MATERIAL AND METHODS

TXNIP and PTP 1B protein retrieval, Ligand preparation

and active site prediction: The crystal structure of the TXNIP and PTP 1B proteins was obtained from the Protein Data Bank of the Research Collaboratory for Structural Bioinformatics (www.rcsb.org/pdb). ADT was used to add hydrogen bonds & Kollman charges to the obtained protein structure. The structures of Petunidin, Hirsutidin, Catharanthine, and Vindoline, as well as the control medication Rosiglitazone, were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov) in canonical SMILES. ChemSketch was used to build and optimise 3D structures. The compound structures were stored as MDL mol files (.mol). The MDL mol files were then translated to PDB format via the Open Babel molecular converter (Boyle et al 2011).

Figure 11: The docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Vindoline calculated by molecular docking study.

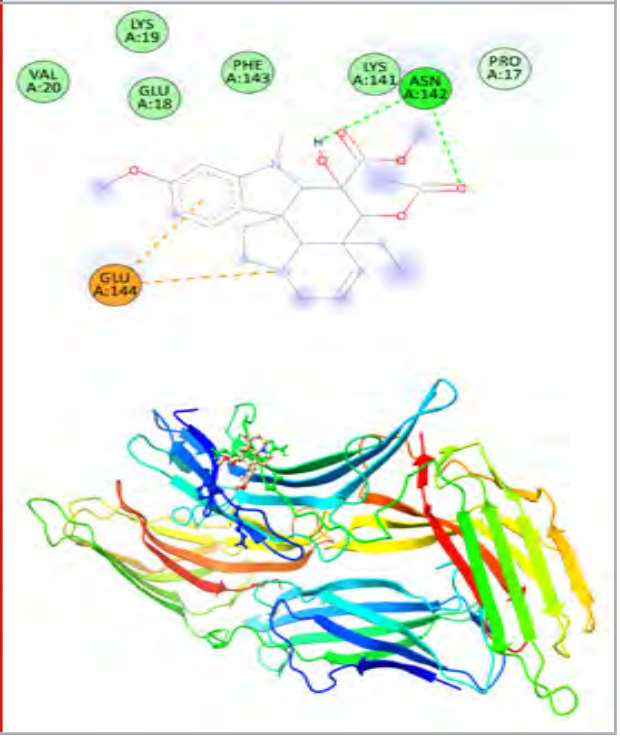
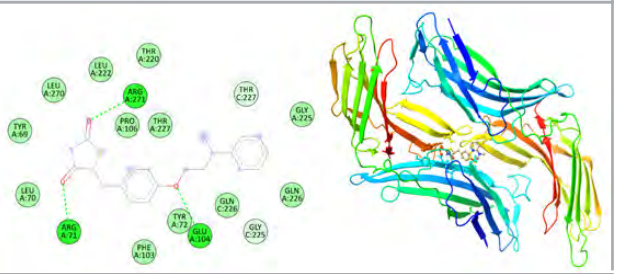


Figure 12: The docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Rosiglitazone calculated by molecular docking study.



Evaluation of Molecular Properties and Ligands ADMET prediction studies for the chemicals: The compounds' physiochemical and pharmacological characteristics were examined using the the "SwissADME" an implement offered by Swiss Institute of Bioinformatics (www.swiss adme.ch) for Properties such as molecule size, rotatable bond, logP, and hydrogen bond donor and acceptor properties were calculated. The selected ligands were examined for membrane permeability, bioavailability, distribution, metabolism, and adsorption (Lipinski's rule of 5) (Lipinski et al 2001, Jarrahpour, et al 2012). Compound pharmacokinetic properties was predicted using the web server PkCSM (unimelb.edu.au)(Pires et al 2015). It expects several parameters based on specific qualities. These factors include absorption, distribution, metabolism, and excretion, which help us determine a drug's pharmacokinetics. Drug absorption can be anticipated using variables such as CaCO2 and skin permeability, intestinal absorption, and P-glycoprotein substrate or inhibitor. The drug's distribution was predicted using factors that include volume distribution (VD), permeability of the central nervous system (CNS), and the blood brain barrier (BBB). Metabolism was determined using the Cytochrome P450 model. The drug's excretion was measured by its total clearance, renal substrate.

Molecular docking: To determine the affinity, the AutoDock Tool was used to dock Petunidin, Hirsutidin, Catharanthine, and Vindoline from C. roseus, as well as the control Rosiglitazone, against TXNIP and PTP 1B proteins (GM Morris et al, 1998). Kohlman charges and polar hydrogen atoms were introduced to the 3D TXNIP and PTP 1B macromolecular structure. The protein-ligand interaction of , Hirsutidin, Petunidin, Catharanthine, Vindoline and the control Rosiglitazone against TXNI and PTP 1B P protein was visualised with Molecular graphics laboratory (MGL)/Chimera X tool.

The structures of ligand under investigation were processed in Chimera to obtain a minimum energy conformer through 10 steepest descent steps followed by 100 conjugate gradient steps. The stabilized ligand conformers were also saved in .pdb format. The protein-ligand docking complexes were performed using AutoDock 4.2 (Forli et al.2016,Morris et al 2009). The LGA method was employed. A grid point spacing of 1.000 Å was set, centered on x= 86, y= 86, and z= 86Å for the TXNIP protein docking. And a grid point spacing of 0.500 Å was set, centered on x= 60, y= 60, and z= 60Å for the PTP1B protein docking. The coordinates of central grid point of maps (-14.025, 1.890, 40.242 Å) for TXNIP protein docking and (-1.591, 62.698, 2.975 Å) was used for PTP1B (2BGD) protein docking.

RESULTS AND DISCUSSION

Molecular properties of the bio actives of Catharanthus roseus & Rosiglitazone: Molecular compound selected for the Phyto chemical from the *Catharanthus roseus* have less than 500 daltons of molecular weight which is in accordance with the Lipinski rule of five and is also comparable with the control drug Rosiglitazone. Octanol- water partition coefficient (log P) was found to be less than 5 for all Phyto compounds used in this study. Number of hydrogen bond acceptors for Petunidin, Hirsutidin, and Rosiglitazone was

calculated to be as 6 and 3 hydrogen bond acceptors for Catharanthine and 8 hydrogen bond acceptors Vindoline. Catharanthine, Vindoline and Rosiglitazone have only one hydrogen bond donor whereas Petunidin and Hirsutidin have 5 and 3 donors bond respectively. Surface Area was least for Petunidin and highest for Vindoline satisfying the Lipinski rule of five for prediction as an ideal drug candidate for treatment of T2D.

ADMET properties of the bio actives of Catharanthus roseus & Rosiglitazone: ADMET properties of the bioactives such as Petunidin, Hirsutidin, Catharanthine, and Vindoline from *Catharanthus roseus* & Rosiglitazone , showed satisfactory results on the various parameters used to evaluate the ADMET properties. Absorption parameter Catharanthine violated the Caco2 permeability as it have 1.114 log cm/s permeabilities which should be not more than 0.90 log cm/s. Intestinal absorption (human) is grater than 30% for all the bioactive such as Petunidin, Hirsutidin, Catharanthine, and Vindoline from *Catharanthus roseus* & Rosiglitazone which showed good results on this parameter. Skin Permeability of Vindoline among all the Phyto chemicals and Rosiglitazone was highest with log Kp cm/h of -3.088 but all the bioactive such as Petunidin, Hirsutidin, Catharanthine, and Vindoline from *Catharanthus roseus* & Rosiglitazone showed violation and are greater than -2.5 log Kp cm/h.

P-glycoprotein substrate was formed by all the bio compounds whereas Rosiglitazone have not formed P-glycoprotein substrate and P-glycoprotein II inhibitor. Only Vindoline had all the P-glycoprotein substrate , P-glycoprotein I inhibitor and P-glycoprotein II inhibitor during PkCSM assessment. VDss (human) was low for Hirsutidin and Rosiglitazone with -0.215 L/kg and – 183 L/kg. Fraction unbound (human) for all the phyto chemical and synthetic drug was considered safe while BBB permeability and CNS permeability was only satisfactory for Catharanthine during the analysis. CYP3A4 substrate was not formed by Petunidin whereas CYP2C9 inhibitior was not produced by Catharanthine and CYP2C9 inhibitior by Hirsutidin. CYP3A4 inhibitior none of the natural or synthetic compound forms CYP3A4 inhibitior at the metabolism parameters of pharmacokinetic. On excretion parameters Total Clearance and Renal OCT2 substrate Catharanthine performs poorly. AMES toxicity was also positive for only Catharanthine while Hepatotoxicity was positive for Rosiglitazone only.

Molecular docking analysis: Binding energy (kcal/mol) and inhibition constant (µM) values of the docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Hirsutidin calculated by molecular docking study was -7.62 kcal/mol and 2.61 µM suggestive of its preference over the synthetic drug Rosiglitazone whose Binding energy was -7.12 kcal/mol with inhibition constant at 6.02 µM . All the bio actives selected from *Catharanthus roseus* performs good in comparison to the Rosiglitazone at parameters like Binding energy (kcal/mol) and inhibition constant (µM) values during Molecular docking analysis of the docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) as shown in table no 4.

All the bio actives selected from *Catharanthus roseus* performs good in comparison to the Rosiglitazone at the parameters like Binding energy (kcal/mol) and inhibition constant (µM) values during Molecular docking analysis of the docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) as shown in table no 4. Binding energy (kcal/mol) and inhibition constant (µM) values of the docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Catharanthine calculated by molecular docking study was -5.8 kcal/mol and 56.12 µM suggestive of its preference over the synthetic drug Rosiglitazone whose Binding energy was -7.12 kcal/mol with inhibition constant at 6.02 µM.

The docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Catharanthine has three van der waals bond at PHE 182, ILE 219 and GLN 262 , two Alkyl bonds at ALA 217 and VAL 49 with a single Pi-Pi Bond at TYR 46, Hydrogen bond at AGR 47 and a salt bridge at ASP 48. The docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Hirsutidin had five conventional hydrogen bond at ASP 48, GLN 262, SER 216 , ARG 221 and GLU 115 followed by Van der waals active forces at ARG 47, GLY 218, ILE 219 and GLY 220. The complex also do have carbon hydrogen bond at ASP 181 and LYS 120, Pi- Pi bond at PHE 182 and TYR 46 , Alkyl bond at ALA 217 & CYS 215 followed by Pi sigma bond at VAL 49.Tyrosine phosphatase PTP1B(2BGD) on docking with Petunidin produces 6 conventional hydrogen bond at ASP 48, SER 216, AGR 221, GLU 115, GLN 262 and ALA 217 followed by Pi sigma bond at VAL 49. The docking complex also have Pi-Pi bonds at TYR 46 and PHE 182.

The complex between Tyrosine phosphatase PTP1B(2BGD) and Petunidin also have five Van Der Waals forces site at GLY 220, GLY 128, ASP181, ARG 47 and LYS 120 with an alkyl bond at CYS 215.Tyrosine phosphatase PTP1B(2BGD) on interaction with Vindoline produces Pi- alkyl bonds at MET 258, ALA 217, VAL 49 AND ILE 219 and a salt bridge at ASP 48. Only one conventional hydrogen bond at ARG 24 with four Van der waals site at ARG 46, PHE 182, ARG 254 and TYR 46. The complex also do have two Carbon Hydrogen bonds at TYR 20 and GLN 262.

The docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Rosiglitazone had a Pi sulphur bond at PHE 182 and Pi-Pi bonds at PHE 182 and TYR 46 . The Interaction studies also reveals that there is also an unfavourable donor at AGR 221 with nine Van Der Waals site of interactions at SER 222, ASP 181, AGR 47, ASP 48, VAL 49, LYS 120, SER 216 and GLN 262. The docking complexes of proteins Tyrosine phosphatase PTP1B(2BGD) with Rosiglitazone had seven conventional hydrogen bonds positioned at GLY 218, GLY 220, ALA 217, ILE 219, CYS 215, GLN 266 and PHE 182.

Van Der Waals interactions were active at TYR 72, GLN 226, GLY 225, THR 227, PRO 106, GLN 107, GLN 226, THR 227, LEU 222, PHE 103, LEU 270, THR 69 and ARG 71 in the docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Catharanthine. The

docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Catharanthine had conventional hydrogen bond at GLU 104 and GLY 225 with Alkyl bond at PRO 106 and Leu 70. Hirsutidin on interaction with TXNIP produces conventional hydrogen bond at GLY 266 , THR 66 and Lys 64 and carbon hydrogen bond at SER 298 and ILE 296. PRO 262, ILE 260, ILE 269, THR 274 was the site for Pi- alkyl bond in the docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Hirsutidin.

Van Der Waals interactions in the docking complexes of protein TXNIP (4LL1) with Hirsutidin were at GLN 65, GLY 297, VAL 272 , ASN 268, SER 267 and AGR 71.Thioredoxin interacting protein ,TXNIP (4LL1) on interactions with Petunidin had Pi Anion bond at ASP 74 with Pi alkyl bond at LEU 77. Conventional hydrogen bonds were represented by THR 227, ASP 74, GLY 102, LYS 100 and LEU 77.Van Der Waals interactions were active at GLN 226, LYS 228, THR 72, PHE 101, LEU 76, THR 231 and VAL 229 in the docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Petunidin.

The docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Vindoline calculated by molecular docking study showed Pi Anion or active charge at GLU 144 with conventional hydrogen bond at ASN 142. There was also Van Der Waals interactions at VAL 20, GLU 18, LYS 19 , PHE 143 and a carbon hydrogen bond at PRO 17. Thioredoxin interacting protein TXNIP (4LL1) on interaction with Rosiglitazone had three conventional hydrogen bonds at ARG 71, GLU 104 and AGR 271 with two Carbon hydrogen bond at THR 227 AND GLY 225. Van Der Waals forces were active at LEU 70, PHE 103, TYR 72, GLN 226, GLY 225, THR 220, LEU 222, LEU 270 and TYR 69.

The docking complexes of proteins Thioredoxin interacting protein TXNIP (4LL1) with Vindoline calculated by molecular docking study showed Pi Anion or active charge at GLU 144 with conventional hydrogen bond at ASN 142. There was also Van Der Waals interactions at VAL 20, GLU 18, LYS 19 , PHE 143 and a carbon hydrogen bond at PRO 17. Thioredoxin interacting protein TXNIP (4LL1) on interaction with Rosiglitazone had three conventional hydrogen bonds at ARG 71, GLU 104 and AGR 271 with two Carbon hydrogen bond at THR 227 AND GLY 225. Van Der Waals forces were active at LEU 70, PHE 103, TYR 72, GLN 226, GLY 225, THR 220, LEU 222, LEU 270 and TYR 69

CONCLUSION

The molecular docking revealed that Hirsutidin had a lower binding energy (-7.62 Kcal/mol) on the TP1B receptor than Catharanthine on the TXNIP receptor (-5.8 Kcal/mol) when compared with synthetic medicines Rosiglitazone which had binding energy (-7.12 Kcal/mol) & (- 4.75 Kcal/mol) on the TP1B and TXNIP receptors. When evaluated using different parameters, the ADMET qualities of bioactive from Catharanthus roseus, such as Vindoline, Hirsutidin, Petunidin, and Catharanthine, as well as Rosiglitazone, demonstrated satisfactory findings. ADMET properties like a AMES toxicity was positive for only Catharanthine whereas on Hepatotoxicity parameters Rosiglitazone was found to be positive .Finally, the predictions indicated that Hirsutidin or Catharanthine might represent a promising lead option for T2D prevention. It is proposed that the current prediction be validated with experimental toxicology & pharmacological assays in the future.

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Data Availability: Data will be available on request.

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Diversity of Riverine Birds in Melghat Landscape, Maharashtra India

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ABSTRACT

Birds are useful bioindicators and provide conducive dispersal pathways and sufficient cover for migrating birds. As there is a lack of data regarding riverine avian diversity, the present study was carried out from November 2022 to December 2023. The study was done along the rivers flowing through the Melghat landscape in the district of Amravati India. The presence of birds in the area was recorded by a line transect and point count method using binoculars and DSLR cameras. A total of 245 birds belonging to 54 families were recorded. Out of which, 72 species from 20 families of water birds were recorded in the riverine zone and 173 species from 34 families of forest birds were recorded in the riverine zone. The majority of the observed species belonged to the Anatidae family followed by Ardeidae and Scolopacidae. Similarly, the Corvidae, Muscicapidae, and Sylviidae families show the maximum number of forest birds. According to the IUCN status, 87% of species associated with water are classified as least concern (LC), 10% as near-threatening (NT), only 3% are vulnerable. Similarly, for forest recorded birds, it is categorized as 98% species of least concern (LC), and only 2% are categorized as near-threatened (NT). Maximum species diversity was recorded with the forest bird associated in the riverine zone (D = 0.991 and H = 4.903), and minimum was recorded with the water bird associated in the riverine zone (D = 0.973 and H = 3.961). The study showed riverine avian diversity and threats in rivers.

KEY WORDS: DIVERSITY, MAHARASHTRA, MELGHAT, NEAR THREATENED, RIVERINE BIRDS.

INTRODUCTION

Riverine ecosystems are crucial habitats for a wide range of species, includes birds. It supports a disproportionately large fraction of its biodiversity while also acted as significant corridors for the movement of plants, animals, and nutrients (Naiman et. al., 1993; Strayer and Dudgeon, 2010). Riparian zones also provide conducive dispersal pathways and sufficient cover for migrating birds, thereby often supported a higher diversity of bird species (Sinha et. al., 2019). The phenomenon of rivers drying up is a global environmental challenge that has far-reaching implications for ecosystems, communities, and water security. Across the world, numerous rivers are experiencing reduced flow and, in some cases, complete drying.

This alarming trend is attributed to a combination of natural and anthropogenic factors, posing serious threats to biodiversity, livelihoods, and the availability of freshwater resources. Various studies have been conducted on the impact of such human actions on the river flow regime (Adib et al., 2016;; Kousali et al., 2022, Adib 2022). Choi et al (2005) examined the effects of the Hapchon dam on regime change in the Hwang River flow in South Korea. They found that the dam construction caused significant downstream changes in the river path. Humans exploiting rivers will reduce discharges and cross-sectional flow, depth, and even flow velocities, dramatically impacting river-dependent habitats (Jiao et al. 2019). That's why understanding the present status of riverine birds is crucial for assessing the impact of changing river conditions on avian biodiversity. Riverine birds are highly dependent on healthy river ecosystems for their survival, as rivers provide critical habitats for nesting, feeding, and breeding.

India hosts 1353 species of birds out of the 10721 total birds in the world, constitutes 13% of the total bird population, and thus is an area of high avian diversity. The bird fauna of India represents 114 families out of the total 249 families in the world. The inventory of birds in the state of Maharashtra comprises 556 species (Mahabal et al., 2005). More than 577 species have been reported from Maharashtra State (Kasambe 2016). Similarly, in Vidarbha, there are a total of 417 species, and in Amravati, there are 392 bird species (Wadatkar et al., 2016, Praveen & Jayapal 2023).

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Documentation of avian diversity in Melghat has been done for the last 100 years. A preliminary checklist of 33 species of birds was prepared by R.T. Jenkin (then DFO, Melghat) and published in 1925 (Nelson 1925). Later, V. B. Sawarkar, then Director of Melghat Tiger Reserve, prepared and published the first comprehensive checklist of the birds of Melghat, which included 252 species. Malabar Pied Hornbill was not listed in the checklist of birds of MTR until 2003. (Wagh et al., 2003). Thereafter, some important sightings occurred, like the Critically Endangered Forest Owlet (Rithe 2003) and the Malabar Pied Hornbill (Kasambe & Wadatkar 2006). Previously, a total of 263 bird species were recorded in the overall Melghat Tiger Reserve (Mahabal, Anil 2005).

Then, in addition to the checklist of birds in Melghat, which went up to 265 species (Wadatkar et al., 2012), Later on, blue-tailed bee eaters breeding by the Tapti River at the boundary of Melghat were observed by Wadatkar et al. (2014). (Wagh et al., 2015) proposed that the preferential route of dispersal for Malabar Pied Hornbill from the Himalayas to the Western Ghats is through the Satpuda Hills in Central India. Nesting of River Lapwings was first recorded in the Tapi River near the Melghat Tiger Reserve (Wagh et al., 2020). The River Lapwing's population in the Vidarbha area is constrained to just a few large rivers; however, the species is presently at risk of extinction.

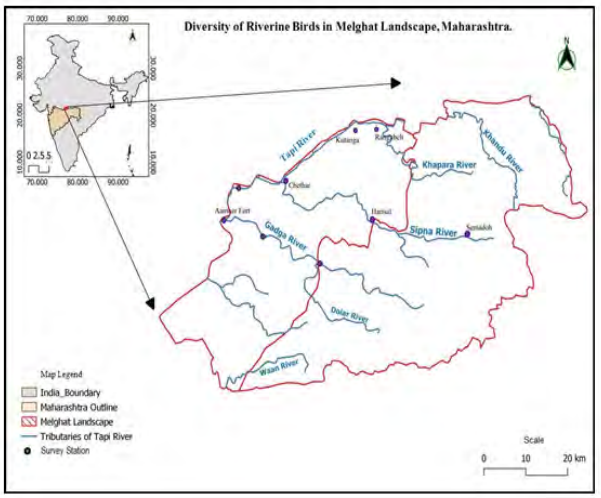
The first ever bird survey of Melghat Tiger Reserve reported a total of 340 bird species (January 2023). During our regular bird watching and surveys being conducted for the River Lapwing and Malabar Pied Hornbill projects, we sighted some species of birds that were not listed in the published checklists of MTR and Amravati district 2016. The results of this study shed light on the importance of conservation and protection of riverine ecosystems for the conservation of avian biodiversity.

MATERIAL AND METHODS

Study Area: The Melghat region is a part of the Satpuda Range of Hills in the Amravati district of central India. This area has dry deciduous forests dominated by teak and bamboo, with excellent tiger habitat. The study area is within latitudes 21°0′15″ to 21°0′45″ N and longitudes 76°00′57″ to 77°00′30″ E at elevations of 312 to 1178 m MSL. It is the largest of the three project tiger programmes in the state. The yearly average temperature is 42.7°C, and the annual rainfall is 1000 mm. The reserve is a catchment area for five major rivers: the Sipna, Gadga, Khandu, Khapra, and Dolar, all of which are tributaries of the Tapti River, which flows through the northern part of Melghat Tiger Reserve and forms the boundary of the Melghat landscape together with the Gawilghur ridge of the Satpura Range. (Fig.1,Table 1)

Survey Methods: Selected locations in the research area will be surveyed over the duration of a study period. Several surveys will be carried out in the summer (March–June) and after the rainy season (July–October) in 2023. A number of trips will be made to the Sipna River at Semadoh, Kolkas, and Harisal. Similarly, surveys of the Tapti river at the specified locations (Chethar, Kutanga, Rangubeli) and Gadga river locations (Dhakna, Kalamkhar, Aamner Fort) and other nearby potential rivers are needed to better understand the status and distribution of river birds.

Figure 1: Map Showing the Rivers of Melghat Landscape with Surveyed Stations, Maharashtra.



A survey has not been conducted on the Khandu River yet. Line transects and point count methods will be used in the riverine ecosystem to determine the diversity and distribution of riverine birds within the river basin. This methodology also helps to determine their abundance in a given research region. Visual scanning is a method of spotting birds primarily based on their visual characteristics and aids in locating their nest during the breeding season. This approach is based on the researcher being able to identify birds visually while recording birds along a predetermined route. For the purpose of searching for riverine birds, visual scanning is the process of scouting riverbanks, mudflats, and sandy areas. Binoculars and spot scopes were used to record observations, and Nikon DSLR cameras equipped with zoom lenses as well as video cameras were used to capture images and videos.

The Garmin GPS was also used to record the latitude and longitude of the broadcast spots. Field survey protocol: The study was carried out during the most active periods, i.e., early morning to mid-morning (6 a.m. to 10 a.m.) and late afternoon to evening (3 p.m. to 6 p.m.). The survey was conducted in the study area with the help of field assistants and local forest staff. To gather more information about the riverine birds in the study area, interviews were performed with locals, tribes, local forest employees, birdwatchers, fishermen, and nature guides. Data sheets and avian science forums were also used, along with field guides for the identification of river birds recorded in water and forest.

Statistical Analysis: Biological diversity indices were calculated to compare riverine sites. Various types of total species diversity indices, including Simpson's diversity index (-D) was used to estimate the biodiversity using the equation: $D = \sum ni\ (ni-1)/\ N\ (N-1)$, Where D = Simpson's

Index of Dominance ni = total number of individuals of a particular species N = the total number of individuals of all species (Simpson, 1949). Similarly, Shannon diversity index was determined by H′= - ∑ (Pi) (ln Pi), in which Pi = Proportion of total species belonging to ith species.The diversity indices were calculated using the software PAST version 4.03. (Table 4)

Table 1: The coordinates of birding station in the rivers of Melghat Landscape.

Rivers	Survey Stations	GPS Coordinates
Tapi River	Chethar	21.6023°N & 76.9085°E
	Kutanga	21.71468°N & 77.0840°E
	Rangubeli	21.71775°N & 77.1401°E
Sipna River	Semadoh	21.4944°N & 77.3122°E
	Kolkas	21.5021°N & 77.1748°E
	Harisal	21.5236°N & 77.1248°E
Gadga River	Dhakna	21.4338°N & 77.0509°E
	Kalamkhar	21.52823°N & 76.813°E
	Aamner fort	21.52814°N & 76.784°E

Figure 2: Family-wise water birds recorded in rivers of Melghat Landscape.

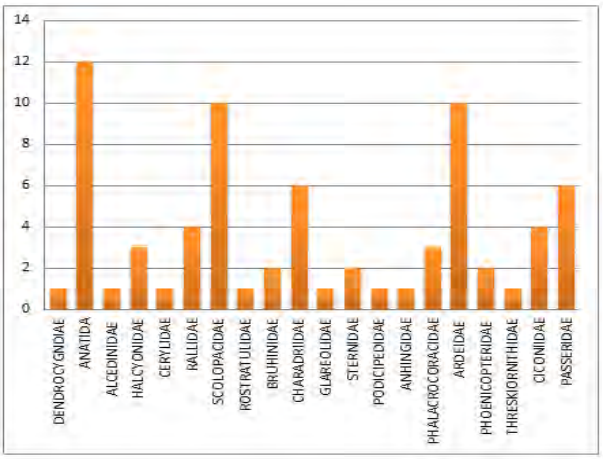
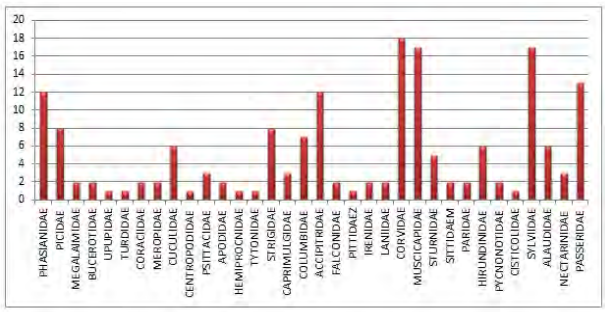


Figure 3: Family-wise forest birds recorded in riverine zone of Melghat Landscape.



RESULTS AND DISCUSSION

In the course of an extensive survey conducted to determine the diversity of riverine birds in the riverine ecosystems of the Melghat Landscape, several key observations were made. The results of the study are as follows: total 245 species of riverine birds belonging to 54 families were recorded. In the riverine ecosystem of the Melghat landscape, a total of 861 individuals from water-recorded bird species were reported, and from forest-recorded birds, 2229 individuals were reported. The study area is richly diversified, with flowing, clean rivers all over the Melghat landscape (Table 4).

Out of which, 72 species from 20 families of water birds were recorded in the riverine ecosystem, and 173 species from 34 families of forest birds were recorded in the riverine ecosystem. Out of the 54 families of birds observed in the course of the study, the majority belonged to the Ardeidae family, followed by the Sylviidae, Phasianidae, Anatidae, Accipitridae, Corvidae, and Charadriidae families, which belong to forest birds (Fig 2 and 3).

A maximum of 12 species were recorded from the Anatidae family of birds in riverine ecosystems, including the Common Teal, Red-crested Pochard, Common Pochard, Indian Spot-billed Duck, Gadwall, Northern Shoveller, Eurasian Wigeon, Ruddy (Brahminy) Duck, Comb Duck (Knob-billed), and Cotton Pigmy Goose. While 10 species from the Scolopacidae family include Black-tailed Godwit, Pintail Snipe, Common Snipe, Common Greenshank, Spotted Redshank, Green Sandpiper, Common Sandpiper, Wood Sandpiper, Little Stint, Temminck's Stint, Also, a maximum abundance of 10 species were recorded from the Ardeidae family, including the Little Egret, Great Egret, Intermediate Egret, Cattle Egret, Grey Heron, Purple Heron, Striated Heron, Indian Pond Heron, Yellow Bittern, and Black Bittern, while the Charadriidae family includes the Red-Wattled Lapwing, River Lapwing, Black-Winged Stilt, Little-Ringed Plover, and Kentish Plover, as well as only one member from the Rostratulidae family, which is the Greater-painted Snipe.

We also noted the conservation status of recorded birds according to the latest updates on the IUCN's list of threatened species (2023), categorised as least concerned (LC), near threatened (NT), and vulnerable (VU), and the red lists of Bird Life International (Tables 2, 3, and Fig. 5 and 6). The IUCN Red List (2023) classified 87% of species as of least concern (LC) and 10% as near-threatening (NT): River Lapwing, Black-tailed Godwit, Great Stone Curlew, River Tern, Darter, Black-headed Ibis, and Painted Stork, where the 2 species that come in the vulnerable category are Asian Woolly-necked Stork and Common Pochard from water recorded birds in the riverine ecosystem of the Melghat landscape. Similarly, for forest recorded birds, it is categorised as 98% species of least concern (LC), and only Malabar Pied Hornbill, European Roller, Alexandrine Parakeet, and Pallas's Fish Eagle are categorised as near-threatened (NT). The study area habitat serves as a suitable habitat for more diversity and species richness in avian fauna.

Observations over different seasons highlighted variations in the composition and abundance of riverine bird species. Certain species were found to be migratory, emphasising the seasonal dynamics and the significance of the Melghat Landscape as a stopover or breeding ground for these birds. Birds migrate by rivers for several compellation reasons. Firstly, rivers serve as natural navigational guides, offering a clear and linear path. Their flow and direction act as visual cues and help birds stay on course during long migrations. This makes the journey more efficient and minimizes the risk of getting lost.

Table 2: Water Birds recorded in riverine habitat of the Melghat landscape.

Common Name	Scientific Name	Family	ST	IUCN status
Lesser Whistling Duck	*Dendro cygnajavanica*	DENDROCYGNDIAE (1)	R	LC
Northern Pintail	*Anas acuta*	ANATIDAE (12)	W	LC
Common Teal	*Anas crecca*		W	LC
Red-crested Pochard	*Rhedonessa rufina*		W	LC
Common Pochard	*Aythya ferina*		W	VU
Indian Spot-billed Duck	*Anas poecilorhyncha*		R	LC
Gadwall	*Mareca strepera*		W	LC
Garganey	*Anas querquedula*		W	LC
Northern Shoveller	*Anas clypeata*		W	LC
Eurasian Wigeon	*Anas penelope*		W	LC
Ruddy (Brahminy) Duck	*Tadorna ferruginea*		W	LC
Comb Duck (Knob-billed)	*Sarkidiornis melanotos*		R	LC
Cotton Pigmy goose	*Nettapus coromandelianus*		R	LC
Common Kingfisher	*Alcedo atthis*	ALCEDINIDAE (1)	R	LC
White-throated Kingfisher	*Halcyon smyrnensis*	HALCYONIDAE (3)	R	LC
Black- Capped Kingfisher	*Halcyon pileata*		R	LC
Stork-billed Kingfisher	*Halcyon capensis*		R	LC
Pied Kingfisher	*Ceryle rudis*	CERYLIDAE (1)	R	LC
White-breasted Waterhen	*Amanrornis phoenicurus*	RALLIDAE (4)	R	LC
Purple Swamphen	*Porphyrio porphyrio*		R	LC
Common Moorhen	*Gallinula chloropus*		R	LC
Common Coot	*Fulica atra*		R	LC
Black-tailed Godwit	*Limosa limosa*	SCOLOPACIDAE (10)	W	NT
Pintail Snipe	*Gallinago stenura*		W	LC
Common Snipe	*Gallinago gallinago*		W	LC
Common Greenshank	*Tringa nebularia*		W	LC
Spotted Redshank	*Tringa erythropus*		W	LC
Green Sandpiper	*Tringa ochropus*		W	LC
Common Sandpiper	*Actitis hypoleucos*		W	LC
Wood Sandpiper	*Tringa glareola*		W	LC
Marsh Sandpiper	*Tringa stagnatilis*		W	LC
Little Stint	*Calidris minuta*		W	LC
Temminck's Stint	*Calidris temminckii*		W	LC
Greater-painted Snipe	*Rostratula benghalensis*	ROSTRATULIDAE (1)	R	LC
Indian Stone-Curlew	*Burhinus indicus*	BRUHINIDAE (2)	R	LC
Great Stone Curlew	*Esacu srecurvirostris*		R	NT
Black-winged Stilt	*Himantopus himantopus*	CHARADRIIDAE (6)	RM	LC
Little-ringed Plover	*Charadrius dubius*		W	LC
Kentish Plover	*Charadrius alexandrinus*		BM	LC
Yellow-wattled Lapwing	*Vanellus malabaricus*		R	LC
River Lapwing	*Vanellus duvaucelii*		R	NT

Red-wattled Lapwing	*Vanellus indicus*		R	LC
Small Pratincole	*Glareola lactea*	GLAREOLIDAE (1)	R	LC
River Tern	*Sterna aurantia*	STERNIDAE(2)	RM	NT
Little Tern	*Sterna albifrons*		BM	LC
Little Grebe	*Tachybaptus ruficollis*	PODICIPEDIDAE (1)	R	LC
Darter	*Achinga melanogaster*	ANHINGIDAE(1)	R	NT
Little Cormorant	*Phalacrocorax niger*	PHALACROCORACIDAE (3)	R	LC
Indian Cormorant	*Phalacrocorax fuscicollis*		R	LC
Great Cormorant	*Phalacrocorax carbo*		R	LC
Little Egret	*Egretta garzetta*	ARDEIDAE (10)	R	LC
Great Egret	*Casmerodius albus*		R	LC
Intermediate Egret	*Mesophoyx intermedia*		R	LC
Cattle Egret	*Bubulcus ibis*		R	LC
Grey Heron	*Ardea cinerea*		R	LC
Purple Heron	*Ardea purpurea*		R	LC
Indian Pond Heron	*Ardeola grayii*		R	LC
Little Green Heron	*Butorides striatus*		R	LC
Yellow Bittern	*Ixobrychus sinensis*		R	LC
Black Bittern	*Ixobrychus flavicollis*		R	LC
Black-headed Ibis	*Threskiornis melanocephalus*	PHOENICOPTERIDAE (2)	R	NT
Red-naped Ibis	*Pseudibis papillosa*		R	LC
Glossy Ibis	*Plegadis falcinellus*	THRESKIORNITHIDAE(1)	R	LC
Painted Stork	*Myeteria leucocephala*	CICONIIDAE (4)	RM	NT
Asian Openbill	*Anastomus oscitans*		W	LC
Asian Woolly-necked Stork	*Ciconia episcopus*		R	VU
Black Stork	*Ciconia nigra*		W	LC
White Wagtail	*Motacilla alba*	PASSERIDAE (6)	W	LC
White-browed Wagtail	*Motacilla maderaspatensis*		R	LC
Citrine Wagtail	*Motacilla citreola*		W	LC
Yellow Wagtail	*Motacilla flava*		W	LC
Grey Wagtail	*Mptacilla cinereal*		W	LC

R- Widespread Resident, W- Widespread Winter Visitor, PV- Passage visitors, RM- Resident Migrant, BM- Breeding Migrant, V- Vagrant or irregular visitors.
IUCN's list of Threatened species (2023), categorized as Least Concerned (LC), Near Threatened (NT) and Vulnerable (VU).

Figure 4: Percentage of resident (R) and migratory (M) Water and Forest birds in study area.

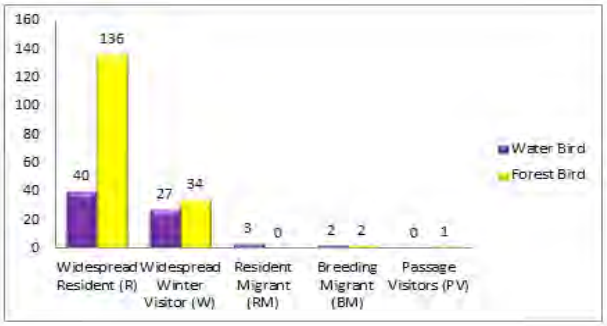
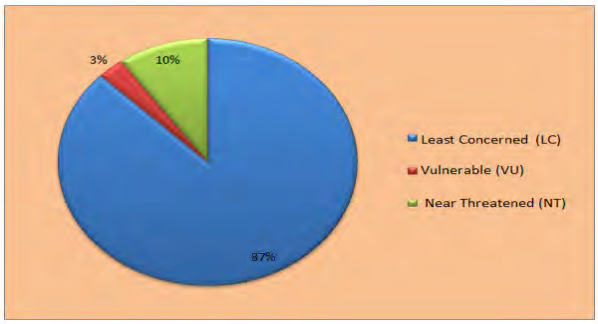


Figure 5: IUCN status of Water birds recorded in rivers in Melghat Landscape.



Secondly, rivers are abundant sources of food, provided migratory birds with a consistent and easily accessible food supply. Fish, insects, and other aquatic organisms thrive in and around rivers, allowed birds to replenish their energy reserves during stopovers. Additionally, the riparian habitats along riverbanks offer suitable resting and roosting sites

for birds. Resting is crucial during migration to conserve energy, and these areas provide shelter and safety, ensured the birds are well-prepared for the next leg of their journey. Lastly, rivers also provide a readily available source of water, essential for birds to drink and bathe in. Migratory birds often pause at rivers to quench their thirst and maintain their plumage, further contributing to their overall well-being during migration.

Table 3: Forest birds recorded in riverine habitat of the Melghat landscape.

Common Name	Scientific Name	Family	ST	IUCN status
Grey Francolin	*Francolinus pondicerianus*	PHASIANIDAE (12)	R	LC
Painted Francolin	*Francolinus pictus*		R	LC
Common Quail	*Coturnix coturnix*		W	LC
Jungle Bush Quail	*Perdicula asiatica*		R	LC
Rain Quail	*Coturnix coromandelica*		R	LC
Barred Buttonquail	*Turnix suscitator*		R	LC
Rock bush Quail	*Perdicula argoondah*		R	LC
Yellow legged Button Quail	*Turnix tanki*		R	LC
Red Spurfowl	*Galloperdix spadicea*		R	LC
Grey Junglefowl	*Gallus sonneratti*		R	LC
Red Jungle fowl	*Gallus gallus*		R	LC
Indian Peafowl	*Pavo cristatus*		R	LC
Eurasian Wryneck	*Jynx torquilla*	PICIDAE (8)	W	LC
Lesser Yellownape	*Picus chlroplus*		R	LC
Yellow-crowned Woodpecker	*Dendrocopos mahrattensis*		R	LC
Golden-rumped Flameback	*Dinopium benghalense*		R	LC
Common Flame Black Woodpecker	*Dryocopus javensis*		R	LC
White-naped Woodpecker	*Chrysocolaptes festivus*		R	LC
Brown-pigmy Woodpecker	*Yungipicus nanus*		R	LC
Lesser yellownape	*Picus chlorolophus*		R	LC
Brown-headed Barbet	*Megalaima zeylanica*	MEGALAIMIDAE (2)	R	LC
Coppersmith Barbet	*Megalaima haemacephala*		R	LC
Indian Grey Hornbill	*Ocyceros birostris*	BUCEROTIDAE(2)	R	LC
Malabar Pied Hornbill	*Anthracoceros coronatus*		R	NT
Common Hoopoe	*Upupa epops*	UPUPIDAE (1)	R	LC
Common Blackbird	*Turdus merula*	TURDIDAE(1)	R	LC
Indian Roller	*Coracias benghalensis*	CORACIIDAE(2)	R	LC
European Roller	*Coracioas garrulus*		W	NT
Green Bee-eater	*Merops orientalis*	MEROPIDAE (2)	R	LC
Blue -tailed Bee eater	*Merops philippinus*		R	LC
Pied Cuckoo	*Clamator jacobinus*	CUCULIDAE (6)	BM	LC
Common Hawk Cuckoo	*Hierococcyx varius*		BM	LC
Indian Cuckoo	*Cuculus micropterus*		R	LC
Grey-bellied Cuckoo	*Cacomantis passerinus*		R	LC
Asian Koel	*Eudynamys scolopaceus*		R	LC
Sirkeer Malkoha	*Phaenicophaeus leschenaultii*		R	LC
Southern Coucal	*Centropus sinensis*	CENTROPODIDAE	R	LC
Alexandrine Parakeet	*Psittacula eupatria*	PSITTACIDAE (3)	R	NT
Rose-ringed Parakeet	*Psittacula krameri*		R	LC
Plum-headed Parakeet	*Psittacula cyanocephala*		R	LC
Little Swift	*Apus affinis*	APODIDAE (2)	R	LC
Asian Palm Swift	*Cypsiurus balasiensis*		R	LC

Crested Tree Swift	*Hemiprocne coronata*	HEMIPROCNIDAE(1)	R	LC
Common Barn Owl	*Tyto alba*	TYTONIDAE (1)	R	LC
Eurasian Eagle Owl	*Bubo bubo*	STRIGIDAE (8)	R	LC
Eurasian Scops Owl	*Otus scopus*		R	LC
Spotted Owlet	*Athene brama*		R	LC
Collared Scops Owl	*Otus scops*		R	LC
Jungle Owlet	*Glaucidium radiatum*		R	LC
Brown Fish- Owl	*Ketupa zeylonensis*		R	LC
Mottled Wood Owl	*Strix ocellata*		R	LC
Forest Owlet	*Heteroglaux blewitti*		R	EN
Indian Nightjar	*Caprimulgus asiaticus*	CAPRIMULGIDAE (3)	R	LC
Indian Jungle Nightjar	*Caprimulgus indicus*		R	LC
Savanna Nightjar	*Caprimulgus affinis*		R	LC
Rock Pigeon	*Columba livia*	COLUMBIDAE (7)	R	LC
Yellow-footed Green Pigeon	*Treronphoenicoptera*		R	LC
Eurasian Collard-Dove	*Streptopeliadecaocto*		R	LC
Red Collard-Dove	*Streptopelia tranquebarica*		R	LC
Spotted Dove	*Spilopelia chinensis*		R	LC
Laughing Dove	*Spilopelia senegalensis*		R	LC
Oriental Turtle Dove	*Streptopelia orientalis*		R	LC
Black-shouldered Kite	*Elanus axillaris*	ACCIPITRIDAE (12)	R	LC
Shikra	*Accipiter badius*		R	LC
Eurasian Sparrow Hawk	*Accipiter nisus*		W	LC
Eurasian Marsh Harrier	*Circus aeruginosus*		W	LC
Pallid Harrier	*Circus macrourus*		W	LC
Short-toed Snake Eagle	*Circaetus gallicus*		R	LC
Pallas's Fish Eagle	*Haliaeetus leucoryphus*		R	NT
Changeable Hawk-Eagle	*Spizhaetus cirrhatus*		R	LC
Black Eagle	*Ictinaetus malayensis*		R	LC
Crested Serpent Eagle	*Spilornis cheela*		R	LC
Oriental Honey Buzzard	*Pernis ptilorhynchus*		R	LC
White-eyed Buzzard	*Butastur teesa*		R	LC
Common Kestrel	*Falco tinnunculus*	FALCONIDAE (2)	W	LC
Lesser Kestrel	*Falco naumanni*		PV	LC
Indian pitta	*Pitta brachyura*	PITTIDAEZ(1)	R	LC
Blue- winged Leafbird	*Chloropsis cochinchinensis*	IRENIDAE(2)	R	LC
Golden Fronted Leafbird	*Chloropsis aurifrons*		R	LC
Bay-backed Shrike	*Lanius vittatus*	LANIIDAE (2)	R	LC
Long-tailed Shrike	*Lanius schach*		R	LC
Rufous Treepie	*Dendrocitta vagabunda*	CORVIDAE (18)	R	LC
House Crow	*Corvus splendens*		R	LC
Large-billed (Jungle) Crow	*Corvus macrorhynchos*		R	LC
Eurasian Golden Oriole	*Oriolus oriolus*		R	LC
Black-hooded Oriole	*Oriolus xanthornus*		R	LC
Large Cuckoo-Shrike	*Coracina macei*		R	LC
Black headed Cuckoo-Shrike	*Coracina melanoptera*		R	LC
White-bellied Minivet	*Pericrocotus erythropygius*		R	LC
Small Minivet	*Pericrocotus cinnamomeus*		R	LC
Black Drongo	*Dicrurus macrocercus*		R	LC
Ashy Drongo	*Dicrurus leucophaeus*		R	LC
White-bellied Drongo	*Dicrurus caerulescens*		R	LC
Greater Racket-tailed Drongo	*Dicrurus paradiseus*		R	LC

White-browed Fantail	*Rhipidura aureola*		R	LC
White-throated Fantail	*Rhipidura albicollis*		R	LC
Asian Paradise-flycatcher	*Terpsiphone paradisi*		R	LC
Common Woodshrike	*Tephrodornis pondicerianus*		R	LC
Common Iora	*Aegithina tiphia*		R	LC
Oriental Magpie Robin	*Copsychus saularis*	MUSCICAPIDAE (17)	R	LC
Indian Robin	*Saxicoloides fulicatus*		R	LC
Orange-headed Thrush	*Zoothera citrina*		R	LC
Blue Rock Thrush	*Monticola solitaries*		W	LC
Malabar Whistling Thrush	*Myophonus horsfieldii*		R	LC
Eurasian Blackbird	*Turdus merula nigropileus*		R	LC
Red-throated Flycatcher	*Ficedula parva*		W	LC
Ultramarine Flycatcher	*Ficedula superciliaris*		W	LC
Tickell's Blue Flycatcher	*Cyornis tickelliae*		W	LC
Verditer Flycatcher	*Eumyis thalassina*		W	LC
Grey-headed Canary Flycatcher	*Culicicapa ceylonensis*		W	LC
Black-naped Monarch	*Hypothymis azurea*		W	LC
Bluethroat	*Luscinia svecica*		W	LC
Black Redstart	*Phoenicurus ochruros*		W	LC
Indian Chat	*Cercomela fusca*		R	LC
Common Stonechat	*Saxicola torquata*		W	LC
Pied Bushchat	*Saxicola caprata*		R	LC
Brahminy Starling	*Sturnia pagodarum*	STURNIDAE (5)	R	LC
Rosy Starling	*Sturnia roseus*		W	LC
Asian Pied Starling	*Gracupica contra*		R	LC
Common Myna	*Acridotheres tristis*		R	LC
Chestnut-tailed Starling	*Sturnia malabarica*		W	LC
Chestnut-bellied Nuthatch	*Sitta castanea*	SITTIDAEM(2)	R	LC
Velvet - fronted Nuthatch	*Sitta frontalis*		R	LC
Great Tit	*Parus major*	PARIDAE(2)	R	LC
Black-lored Tit	*Parus xanthogenys*		R	LC
Dusky Craig Martin	*Hirundo concolor*	HIRUNDINIDAE (6)	R	LC
Plain Martin	*Riperia paludicola*		R	LC
Barn Swallow	*Hirundo rustica*		W	LC
Wire-tailed Swallow	*Hirundo smithii*		R	LC
Red-rumped Swallow	*Hirundo daurica*		R	LC
Streak-throated Swallow	*Hirundo fluvicola*		R	LC
Red-vented Bulbul	*Pycnonotus cafer*	PYCNONOTIDAE (2)	R	LC
Red -whiskered Bulbul	*Pycnonotus jocosus*		R	LC
Zitting Cisticola	*Cisticola juncidis*	CISTICOLIDAE (1)	R	LC
Jungle Prinia	*Prinia sylvatica*	SYLVIIDAE (17)	R	LC
Plain Prinia	*Prinia inornate*		R	LC
Ashy Prinia	*Prinia socialis*		R	LC
Grey-breasted Prinia	*Prinia hodgsonii*		R	LC
Oriental White-eye	*Zosterops palpebrosus*		R	LC
Blyth's Reed Warbler	*Acrocephalus dumetorum*		W	LC
Lesser Whitethroat	*Sylvia curruca*		W	LC
Clamorous Reed Warbler	*Acrocephalus stentoreus*		W	LC
Booted Warbler	*Hippolais caligata*		W	LC
Greenish Warbler	*Phyloscopus trochiloides*		W	LC
Sulphur-bellied Warbler	*Phylloscoppus griseolus*		W	LC
Tawny-bellied Babbler	*Dumetia hyperythra*		R	LC
Common Tailor Bird	*Orthotomus sutorius*		R	LC

Yellow-eyed Babbler	*Chrysomma sinense*		R	LC
Large Grey Babbler	*Turdoides malcolmi*		R	LC
Jungle Babbler	*Turdoides striatus*		R	LC
Common Babbler	*Turdoides caudatus*		R	LC
Indian Bush Lark	*Mirafra erythroptera*	ALAUDIDAE (6)	R	LC
Ashy-crowned Sparrow Lark	*Eremopterix griseus*		R	LC
Sykes's Lark	*Galerida deva*		R	LC
Singing Bushlark	*Mirafra cantillllans*		W	LC
Rufous-tailed Lark	*Ammomanes phoenicura*		R	LC
Greater Short-toed Lark	*Calandrella brachydactyla*		W	LC
Purple-rumped Sunbird	*Leptocomazeylonica*	NECTARINIDAE (3)	R	LC
Purple Sunbird	*Cinnyris asiaticus*		R	LC
Thick-billed Flowerpecker	*Dicaeum agile*		R	LC
Paddyfield Pipit	*Anthus rufulus*	PASSERIDAE (13)	W	LC
Tawny Pipit	*Anthus campestris*		W	LC
Tree pipit	*Anthus trivialis*		W	LC
House Sparrow	*Passer domesticus*		R	LC
Chestnut-shouldered Petronia	*Petronia xanthocollis*		R	LC
Baya Weaver	*Ploceus philippinus*		R	LC
Red Avadavat	*Amandava amandava*		R	LC
Indian Silverbill	*Euodice malabarica*		R	LC
Scaly-breasted Munia	*Lonchura punctulata*		R	LC
Crested Bunting	*Melophus lathami*		R	LC
Black-headed Bunting	*Emberiza melanocephala*		W	LC
Red-headed Bunting	*Emberiza bruniceps*		W	LC
Grey-necked Bunting	*Emberiza buchanani*		W	LC

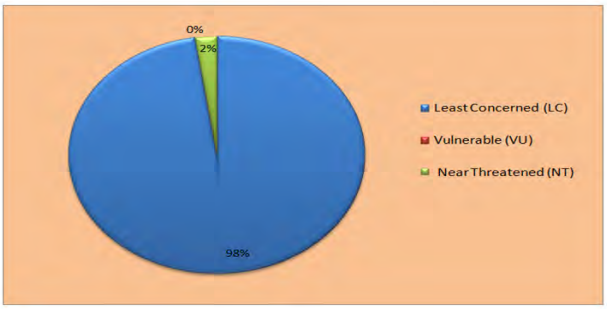
R- Widespread Resident, W- Widespread Winter Visitor, PV- Passage visitors, RM- Resident Migrant, BM- Breeding Migrant, V- Vagrant or irregular visitors, from Melghat Landscape.
IUCN's list of Threatened species (2023), categorized as Least Concerned (LC),
Near Threatened (NT) and Vulnerable (VU).

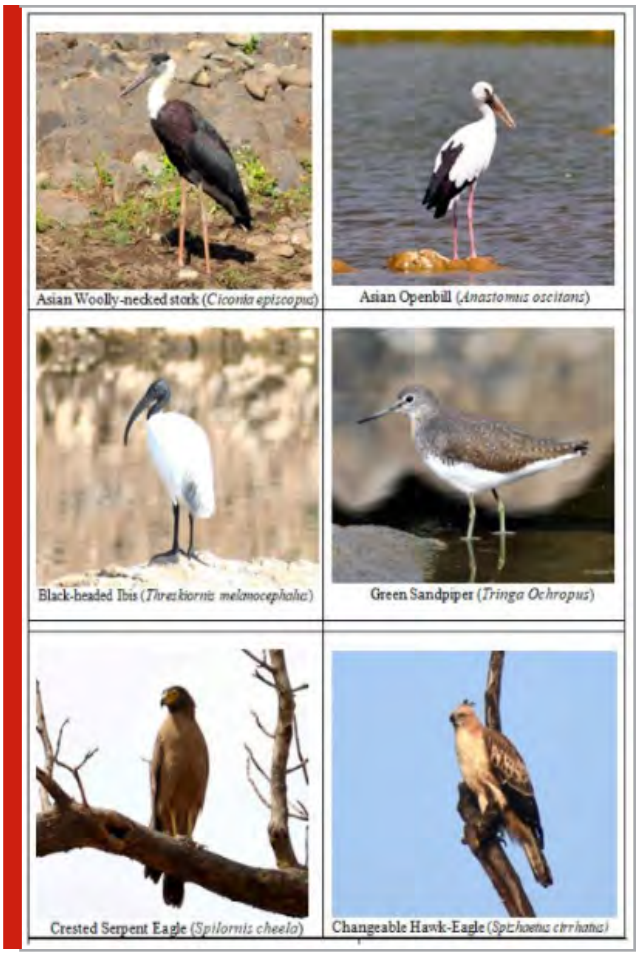
Table 4: Summary of Diversity indices of water birds and forest birds recorded in rivers of Melghat Landscape.

Observations	Water birds recorded in river	Forest birds recorded in river
Species numbers	72	173
Total Individuals	861	2229
Simpson Diversity Index [D]	0.973	0.991
Shannon Diversity Index [H]	3.961	4.903
Menhinick Index	2.42	3.604
Margalef Index	10.36	21.93
Evenness	0.7396	0.7921
Berger-Parker	0.06504	0.03146
Dominance	0.0247	0.0009
Fisher alpha	18.35	42.83

In the riverine survey of Melghat rivers, all the recorded species were categorised according to their presence in the study area. Where Widespread Resident (R) constitutes 40 species that account for 56%, Widespread Winter Visitor (W) includes 27 species that account for 37%, Breeding Migrant (BM) includes Kentish Plover, Little Tern, and Resident Migrant (RM), 3 species that are Painted Stork, Black-Winged Stilt, and River Tern from the water recorded birds of the riverine ecosystem of Melghat landscape (Fig.4).

Figure 6: IUCN status of forest birds recorded in rivers in Melghat Landscape.





Similarly, Widespread Resident (R) comprises 136 species that account for 79%, Widespread Winter Visitor (W) includes 34 species that account for 20%, Breeding Migrant (BM) includes Pied Cuckoo, Common Hawk Cuckoo, and Passage Visitors (PV) have Lesser Kestrel from the forest recorded birds of the riverine ecosystem of Melghat landscape (Fig.4).

Indicator bird species were chosen according to their ecological affinity for one of the four major wetland habitat types (marshes, wet meadows, shrub swamps, and treed swamps), their sensitivity to hydrological conditions (depth of surface water and fluctuations of water level) (Weller,

1999), and their nesting strategy (i.e., on ground near shoreline, on floating vegetation, or attached to vegetation above water or on ground) (Gibbs et al., 1991; Steen and Gibbs, 2002). The analyses presented here are restricted to a limited selection of indicator species distributed according to the four major wetland habitat types, riverine habitat types, the vulnerability of their nests, and the nature of the statistical relationship with hydrological variables, (Sinha et. al 2019).

Riverine birds are birds that are found in and around rivers, streams, and other bodies of freshwater. Some indicator species of riverine birds that are located as indicator species include the Green Sandpiper, River Tern, River Lapwing, Malabar Whistling Thrush, Pied Kingfisher, Stork Billed Kingfisher, Little Egret, Indian Cormorant, etc. These species were chosen based on their association with riverine habitats, sensitivity to changes in hydrological conditions, and nesting strategies.

The survey revealed a rich diversity of riverine birds in the Melghat Landscape, included various species such as egrets, kingfishers, herons, ducks, and waders. The presence of multiple species indicated a healthy and diverse avian community dependent on riverine ecosystems. The diversity index, species evenness, and species abundance were studied. In the water bird recorded with riverine ecosystem study area, various diversity indices, as mentioned, showed the result like Simpson Diversity Index is 0.973, Shannon Diversity Index is 3.961, Menhinick Index is 2.42, Margalef Index is 10.36, Berger-Parker is 0.06504, and evenness is 0.7396. Similarly, in forest birds recorded in riverine ecosystem areas, the Simpson Diversity Index is 0.991, the Shannon Diversity Index is 4.903, the Menhinick Index is 3.604, the Margalef Index is 21.93, the Berger-Parker is 0.03146, and evenness is 0.7921. Whereas abundance on the river of Melghat landscape reservoir is 3086 (Table 4).

The consistent presence of both the Pied kingfisher and the White-throated kingfisher across all riverine habitats in the Melghat landscape highlights the ecological adaptability and widespread distribution of these avian species in the region. The presence of the Black-capped kingfisher exclusively observed along the Dolar River underscores its ecological significance and uniqueness. The population of the Black-capped Kingfisher in this specific location is experiencing a decline. The potential sites for the River Lapwing as a hotspot are Chethar, Kutanga, and Rangubeli, and similar sites for the Stork-billed Kingfisher are Kolkas, Chaurakund, Rangubeli, Semadoh, and Harisal.

The observations of this survey contributed to significant bird sightings like River Lapwing and Stork-billed Kingfisher from the Tapi River and Sipna River, which had significant results at Rangubeli, Kutanga, Chethar, Amner Fort, Semadoh, and Harisal, respectively. The Forest Owlet (Heteroglaux blewitti), a critically endangered species of Owl that was thought to be extinct for over a century, was observed at Churni Nala Chaurakund, which is ultimately part of the Sipna River. The distribution of River Lapwing (Vanellus duvaucelii) is confined to the Tapi River; it is not observed in other rivers of the Melghat landscape.

The presence of the Green Sandpiper in the riverine habitats of the Tapti and Sipna rivers indicates the significance of these water bodies as suitable environments for this particular bird species. Likewise, Malabar Pied Hornbill, Painted Stork, Black Stork, Asian Woolly-necked Stork, Eurasian Eagle Owl, Brown Fish-Owl, Great Stone Curlew, Black-headed Ibis, Glossy Ibis, European Roller, Brown Crake, etc. contributed to the scientific understanding of riverine birds in the Melghat Landscape but also provided a foundation for informed conservation actions. This study has established the framework for focused conservation measures aimed at preserving the high biodiversity of riverine habitats in the Melghat Landscape by extensively documenting the condition and variety of these birds.

Primary threats to riverine ecosystems include direct and indirect threats, such as sand mining and illegal fishing with explosive material and feral dog movements which can disrupt critical nesting and foraging sites for riverine birds. Anthropogenic activities, such as water extraction for agriculture can alter water levels, ecological parameters, and seasonal events, such as seasonal crop framing in river banks, affecting their survival and health.

CONCLUSION

The study revealed that the Melghat landscape is a unique habitat for a diverse range of riverine birds. The high diversity of 245 riverine bird species in the study area highlights the importance of the riparian zones as a crucial element of the natural system. The majority of the observed species belonged to the Ardeidae family, with the maximum number of Little Egrets, Little Cormorant, and River Tern as it is an indicator species of riverine ecosystems. These are opportunistic feeders and consume a variety of aquatic organisms, such as fish, amphibians, crustaceans, and insects. Their feeding activities can help to regulate the population of prey species and thus maintain the balance of the riverine ecosystem, which is consistent due to clean water in riverine systems.

These findings suggest that the Tapi River is a crucial habitat for these near-threatened species, River Lapwing, and its distribution is restricted to the only Tapti River, which is a large and flowing river. Such a flowing river serves as a lifeline for riperine birds. The Stork-billed Kingfisher and Black-capped Kingfisher are important in Melghat for their roles in maintaining ecological balance by controlling fish and insect populations, serving as indicators of healthy riparian ecosystems, and contributing to the region's biodiversity and ecotourism appeal.

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Conflict of Interest: Authors declare no conflict of interest.

Data Availability: Data will be available on reasonable request, made to the corresponding author.

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