Assessment of liposome encapsulated *Morinda tinctoria* Roxb. Leaves for wound healing activity

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The thesis submitted in fulfillment of the requirements for the Degree of Master of Science (M.Sc.) in Zoology

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ABBERIVATION

WHO - World Health Organization

WHA- Wound healing activity

TIM - Traditional Indian Medicine

MCM - Medical Counter Measures

MRSA - Methicillin Resistant Staphylococcus aureus

EESG - Ethanol Extract of Morinda tinctoria

DPPH - 2, 2-diphenyl-1-picryl-hydrazyl-hydrate

ADME- Absorption, Distribution, Metabolism and Excretion

INTRODUCTION

About 65% of total global population remains dependent on traditional medicines for their primary healthcare. Herbs are occupying a comeback and an Herbal Renaissance is blooming across the world (Roy *et al*., 2018). They have been evidently prized for their medicinal, flavoring and aromatic qualities for centuries, yet for a while they were over shadow by synthetic products of modern civilization (Ullah *et al*., 2020).

Herbal medicines which formed the basis of health care throughout the world since the earliest days of mankind are still widely used, and have considerable importance in international trade. Recognition of their clinical, pharmaceutical and economic value is still growing, although this varies broadly between countries. Medicinal plants are important for pharmacological research and drug development, not only when plant constituents are used directly as remedial agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds(Newman and Cragg, 2016). Regulation of development and exportation is therefore essential, together with international cooperation and coordination for their conservation so as to ensure their availability for the future. Despite the use of herbal medicines over many centuries, only a relatively small number of plant species has been studied for possible medical applications. Safety and efficacy data are available for an even smaller number of plants, their extracts and active ingredients and preparations containing them.

We all know that wounds are inescapable events in life. Wounds may arise due to physical, chemical or microbial agents. Wound healing is the natural process which leads to restore the structural and functional integrities of injured tissues or it is a complex and dynamic process of replacing devitalized and missing cellular structures and tissue layers. The wound healing process can be divided into three or four distinct basic phases. Inflammatory, fibroblastic or proliferation, and maturation or remodeling constitutes the three-phase division (Gilmore *et al*., 1991; Mercandetti *et al*., 2017). It involves several biochemical and cellular pathways, in order to repair the lesions and to restore the physiological conditions. Fortunately, the human

body has the inbuilt capacity to promote this repairing process (Maver *et al*.,2015). However, there can be impairment of this sophisticated repairing process leading to chronic or non-healing wounds, which may result in severe clinical complications or even patient death.

Many drugs are known to impair wound healing. Chemotherapeutic drugs are used in cancer are the largest group well known to delay wound repair. Systemic glucocorticoids inter fere normal healing process by reducing collagen synthesis and fibroblast proliferation. Once having realized their sources and adverse effects, people are going to nature with hopes of safety and security. Based on this, an alternative medicine with little or even no side effects derived from herbs or other natural materials are needed. The rich treasure of herbal drugs is forming a boon for our society. Plant derived compounds, apart from their nutritive values, could serve as important therapeutic weapons to fight various human and animal diseases, thereby making them indispensable in traditional medicine for treating a number of diseases. Plant drugs, popularly known as herbal medicines have since been unabatedly used to those various diseases. The major challenge is to protect traditional knowledge and will prove to be a beneficial asset to our human surrounding. For all the ailments herbal formulations are proved to be effective without any side effects commonly seen with allopathic drugs (BanoMirza *et al*., 2015).

Phytochemical are bioactive non nutrient components of plants, commonly found in the wound healing, that may have beneficial (or harmful) health effects and include flavonoids, glucosinolates, sulfur compounds, saponins, monoterpenes, sesquiterpene, capsaicinoids, and cyprinoids (Chaudhary *et al*., 2018). They are often used for primary disease prevention or as adjuncts to conventional therapies (Ulbricht and Chao, 2010). Knowing that phyto chemicals are a key source of potential anticancer compounds, the NCI established the Natural Products Branch (NPB) in 1960 as a depository for crude natural product materials that are screened for pharmacological activity. Over 70,000 plant samples are currently in the NCI Natural Products Repository, and ongoing efforts are increasing the number of compounds that may be screened for wound healing potential (Sarkar, 2010).

Several phytoconstituents such as triterpenes, alkaloids, and polyphenols show antioxidant and antimicrobial effects and are able to promote one or more mechanisms of the reparative process (Somashekar, 2013). Accordingly, numerous plant extracts have been employed to promote wound healing with a high degree of success (Fikru*et al*., 2012). Many

wound healing medicinal plants have been investigated to possess antioxidant properties. In other dimensions, numerous studies conducted over the years showed the great potential of plants in promoting wound healing, by virtue of their high contents in antioxidant properties. Studies reported that Morinda species are found to have wound healing activity.

There are different Morinda species found worldwide as *Morinda citrifolia* Linn. *Morinda elliptica* Ridley, *Morinda tinctoria* Heller and *Morinda tinctoria* Roxb of which, *M. umbellate L. M. citrifolia L.* and *M. tinctoria* Roxb. are best known and most widely cultivated in South India and are medicinally used in India and China (Kritikar and Basu, 1935). *Morinda tinctoria* Roxb. (*M. tinctoria* Roxb.) Is an evergreen shrub native to southern Asia, upper and lower Burma, Bengal, Bihar, central provinces and in the Deccan westwards to the eastern slopes of the ghats in India. The genus Morinda grows wild and is widely distributed in southern India. *M. tinctoria* Roxb. Commonly known as Aal or Indian mulberry belongs to the family Rubiaceae. *M. tinctoria* Roxb. is an evergreen shrub or small tree growing to 5 - 10 m tall. The different parts of *M. tinctoria* Roxb. have been used traditionally for various purposes. South Indian ancestors realized the therapeutic value of *M. tinctoria* Roxb. and used it in traditional medicinal systems, lack of proper documentation resulted in loss of that knowledge (Narayanasamy *et al*., 2006). Indian mulberry is reportedly used by the African aborigines medicinally. Fruits, flowers, leaves and heartwood of *M. tinctoria* Roxb. Have been used in treating several diseases, in Ayurveda (Kritikar and Basu, 1935). So, an attempt has been made to review various aspects of its traditional uses, phytoconstituents and therapeutic potential of various parts of this plant.

M.tinctoria Roxb. leaves contain a set of pharmaceutically important phyto chemicals like octoanic acid, potassium, vitamin-C, terpenoids, scopoletin, flavones, glycosides, lineoleicacid, anthraquinones, morindone, rubiadin and alizarin (Krishnaiah *et al*., 2007). Anthraquinones are reckoned as one of the major constituents of the Morinda genus, showing antitumor promoting, antioxidant, anti-inflammatory, purgative and astringent activities(Bhakta and Siva, 2012). This plant serves an excellent medicine against arthritis, diarrhea, viral infection, astringent, gastric ulcer, liver diseases and diabetes (Chopra *et al*., 1956).

Molecular docking is the *Insilico* approach where the protein and ligand are used to find the best interactions between them. Hence, the results from *In silico* studies could be used to find the

relevant information before conducting *in vitro* and *in vivo* studies. *In siilco* prediction systems are cheaper, rapid, and reproducible and have low compound synthesis requirements. They can undergo constant optimization and have potential to replace the use of animals (Valerio-Jr, 2009). The application of *Insilico* methods in herbal sciences is used for the detection of phytochemical compounds bearing known genotoxicity reducing activity. They can help to clarify compounds which are responsible for a proven effect. In the present study, selected wound healing activity of proteins from Morinda plant are supposed to dock with particular receptors which offer a great hope in the identification of lead compounds for the treatment of wound healing activity.

Liposomes have been considered promising and versatile drug vesicles. Compared with traditional drug delivery systems, liposomes exhibit better properties, including site-targeting, sustained or controlled release, protection of drugs from degradation and clearance, superior therapeutic effects, and lower toxic side effects. Given these merits, several liposomal drug products have been successfully approved and used in clinics over the last couple of decades.

Liposomes are the spherical shaped small vesicles that can be produced from cholesterols, nontoxic surfactants, sphingolipids, glycolipids, long chain fatty acids and even membrane proteins. Phospholipids spontaneously form a closed structure when dissolved in water with internal aqueous environment bounded by phospholipids bilayer membranes, this vesicular system is called as liposome. Liposomes are the drug carrier loaded with great variety of molecules such as small drug molecules, proteins, nucleotides and even plasmids.

Liposomes has become the versatile tool in Biology were discovered about 40 years ago. It was first described by British hematologist Dr Alec D Bangham FRS in 1961at the Babraham Institute, in Cambridge. Liposome can be formulated and processed to differ in size, composition, charge and lamellarity. In 1970s the clinical potential of liposomes as a vehicle for replacement therapy in genetic deficiencies of liposomal enzymes was first demonstrated. In 1970s and 1989s considerable progress was made in the field of liposome stability leading to long circulation times of liposomes after intravenous administration resulting in the improvement in bio-distribution of liposome. The clinical applications of liposomes are well known. The initial success achieved with many liposome-based drugs has fuelled further clinical investigations.

The present investigation has a broader objective of comprehensive evaluation of the antioxidant activity of the *M.tinctoria* Roxb. in three different model systems. Using molecular docking studies, an attempt has been made to identify the active principle behind wound healing that may suggest its use as a potential medicine against breast wound healing. The *Invitro* assays were carried out using the cultured breast cancer cell lines to understand possible mechanism for antimicrobial activity of wound healing. Further, Sprague was used as the plant model for the *In vivo* studies to understand wound healing principle by exploring wound healing activity, biochemical and antioxidant parameters.

There is a promising future of medicinal plants as there are about half million plants around the world, and most of them are not investigated yet for their medical activities and their hidden potential of medical activities could be decisive in the treatment of present and future studies.

Although there are studies related to the wound healing activity of *M.tinctoria*Roxb., the assessment of liposome encapsulated *M. tinctoria* Roxb. leaves for its efficacy to heal wounds has not yet reported. Hence the present study is designed to assess liposome encapsulated *M. tinctoria* Roxb. leaves for wound healing activity and to conduct *In silico* molecular docking studies using bioactive compounds to evaluate their potential efficacy against wound healing proteins.

Objectives:

Considering all the above information, the present investigation was undertaken with the following objectives.

- To evaluate the phytochemical constituents present in *M. tinctoria* Roxb leaves.
- To assess the antioxidant potential of ethanolic extract of *M.tinctoria* Rox*b.* leaves
- To assess the efficacy of liposome encapsulated using *M. tinctoria* Roxb. Leaves
- To analyse the antimicrobial potential of liposome synthesized using *M. tinctoria* Roxb. Leaves.
- To understand the *interaction of* compounds from *M. tinctoria* Roxb. leaves with proteins responsible for the pathogenesis of the wound using molecular docking.

REVIEW OF LITERATURE

The review of literature pertaining to the study **"Assessment of liposome encapsulated *Morinda tinctoria Roxb.* leaves for Wound healing activity**" was explored and its review is presented in the following pages.

2.1 Wound healing activity- Overview

A wound is defined as damage or disruption to the normal anatomical structure and function. This can range from a simple break in the epithelial integrity of the skin or it can be deeper, extending into subcutaneous tissue with damage to other structures such as tendons, muscles, vessels, nerves, parenchymal organs and even bone.

Wounds can arise from pathological processes that begin externally or internally within the involved organ. They can have an accidental or intentional etiology or they can be the result of a disease process. Wounding, irrespective of the cause and whatever the form, damages the tissue and disrupts the local environment within it. A physiological response to the noxious factor results in bleeding, vessel contraction with coagulation, activation of complement and an inflammatory response (Galvão *et al.,* 2018). Normal wound healing is a dynamic and complex process involving a series of coordinated events, including bleeding, coagulation, initiation of an acute inflammatory response to the initial injury, regeneration, migration and proliferation of connective tissue and parenchyma cells, as well as synthesis of extracellular matrix proteins, remodeling of new parenchyma and connective tissue and collagen deposition. Finally, increasing the wound strength takes place in an ordered manner and culminates in the repair of severed tissues.

Wound healing begins at the moment of injury and involves both resident and migratory cell populations, extracellular matrix and the action of soluble mediators. The mechanisms underlying the processes described above involve: (i) inflammatory mediators and growth factors; (ii) cell–cell and cell–extracellular matrix interactions that govern cell proliferation, migration and differentiation; (iii) events involved with epithelialization, fibroplasias and

angiogenesis; (iv) wound contraction; and (v) remodeling (Alariba, 2022). These mechanisms are initiated at the time of physical injury and proceed continuously throughout the repair process.

Despite the fact that the processes of repair begin immediately after an injury in all tissues and that all wounds go through similar phases of healing, specialized tissues such as liver, skeletal tissue and the eye have distinctive forms of regeneration and repair and follow separate pathways. Additionally, there are differences between tissues in terms of the time required to complete regeneration. Wound healing time can be diverse and some wounds may take up to a year or more to heal completely. A completely healed wound is defined as one that has been returned to a normal anatomical structure, function and appearance of the tissue within a reasonable period of time. Most wounds are usually the result of simple injuries; however, some wounds do not heal in a timely and orderly manner. Multiple systemic and local factors may slow the course of wound healing by causing disturbances in the finely balanced repair processes, resulting in chronic, non healing wounds (Velnar *et al.*, 2016).

Wounds that repair themselves and that proceed normally by following a timely and orderly healing pathway, with the end result of both functional and anatomical restoration, are classified as acute wounds (Visha and Karunagaran, 2019). The time course of healing usually ranges from 5 to 10 days, or within 30 days. Acute wounds can be acquired as a result of traumatic loss of tissue or a surgical procedure. For example, an operation to remove a soft tissue tumour located in the skin and underlying parenchyma can sometimes result in a large albeit non contaminated wound that cannot be healed by primary intention, due to the large defect within the tissue. Traumatic wounds are also frequently encountered. They may involve only the soft tissue or they might be associated with bone fractures. These combined injuries have been classified by the classification system of the AO Foundation association for the Study of Internal Fixation), which is one of the most comprehensive and widely used. Included within this classification system are closed and open fractures with the assessment of skin, muscle, tendon and neurovascular injuries. A benefit of the AO Foundation's classification system is that the extent of damage to muscles and tendons is taken into account, as it determines the prognosis of the injured limb.

2.2 Natural plant product research

Since prehistoric times, humans have used Natural Products (NPs), such as plants, animals, microorganisms, and marine organisms, in medicines to alleviate and treat diseases. According to fossil records, the human use of plants as medicines may be traced back at least 60,000 years (Yuan and Haidan, 2016; Shi *et al.*, 2010). The use of natural products as medicines must, of course, have presented a tremendous challenge to early humans. It is highly probable that when seeking food, early humans often consumed poisonous plants, which led to vomiting, diarrhea, coma, or other toxic reactions—perhaps even death. However, in this way, early humans were able to develop knowledge about edible materials and natural medicines (Yuan and Haidan, 2016). Subsequently, humans invented fire, learned how to make alcohol, developed religions, and made technological breakthroughs, and they learned how to develop new drugs.

NPs have evolved over millions of years and acquired a unique chemical diversity, which consequently results in the diversity of their biological activities and drug-like properties. Therefore, even before the rise of the modern chemical pharmacology, NPs have been used for centuries as components of traditional medicines, in particular as active components of herbal remedies. Nowadays, some of the traditional healing practices, such as Indian Ayurveda, traditional Chinese medicine or African herbal medicines, remain the primary treatment option for many people across the world, due to economic reasons, to personal beliefs or to the difficulty in accessing pharmaceutical products. In modern pharmacology too, NPs have become one of the most important resources for developing new lead compounds and scalds (Newman and Cragg, 2016; Khalifa *et al.*, 2019; Bano Mirza *et al.*, 2015).

The World Health Organization listed between 1999 and 2009 a list of over 21 000 plants used for medicinal purposes all over the world (WHO, 2009). This effort was made for proper identification of safe plants, as it is estimated that plant-based traditional medicines are used by 60% of the world's population (Polur *et al.*, 2011). In addition to efforts to establish formal, DNA-based identification of such plants for wider use (Palhares *et al.*, 2015), collections of medicinal plant species, and in particular of phytochemical, NPs produced by plants, associated to their therapeutic activities and physicochemical properties are being established around the world. This is particularly the case in Asia and Africa, where traditional medicines remain an important part of everyday life for cultural, traditional and economic reasons.

Traditional Chinese Medicine (TCM) is naturally part of the Chinese public health system (Yuan *et al.,* 2016). It is therefore coherent that in this country the scientific study of natural compounds from plants used in TCM is very advanced and is receiving strong governmental support, and they have developed a plethora of databases containing NPs, their sources and effects. The biggest database containing NPs used in TCM is TCM@ Taiwan (Chen, 2011). It contains over 58,000 entries and is directly feeding iS MART (Chang *et al.*, 2011), an integrated cloud computing web server for online virtual screening, evolution studies and drug design.

In addition to this, there are several other, smaller, databases for NPs, TCM that can be cited, such as the Chinese Ethnic Minority Traditional Drug Database (CEMTDD) (Huang *et al.,* 2015), that is maintained, but not updated and contains 4000 NPs, the Chinese Traditional Medicinal Herbs Database (CHDD) (Qiao *et al.,* 2002), not maintained anymore, but according to the publication contained over 30,000 entries, now not accessible and probably lost for the scientific community. Some other databases containing phyto chemicals and other active compounds used in TCM can be cited, such as the Comprehensive Herbal Medicine Information System for Cancer (CHMIS-C) (Fang *et al.,* 2005) that is not maintained anymore, the En cyclo podia of Traditional Chinese Medicine (ETCM) (Xu *et al.,* 2019), that is maintained but the chemical structures it contains are not easily retrievable, the database of medicinal materials and chemical compounds in Northeast Asian TM (TM-MC) (Kim *et al.,* 2015), which is maintained, updated, but no structures but contains precise plant species for all compounds, the Traditional Chinese Medicine Integrative Database (TCMID) (TCMID, 2019), maintained, but not updated anymore, The Traditional Chinese Medicine Systems Pharmacology database and analysis platform (TCMSP) (Huang *et al.,* 2014), that is also not maintained anymore but used to contain over 29,000 NPs. One can quickly realize that there is a lot of databases that focus on chemical compounds used in TCM, and creators of the latter recognize it: there is even a database called "Yet Another Traditional Chinese Medicine Database" (YaTCM) Zhao *et al.,* 2018) that was published in 2018.

2.3 Traditional medicine

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. In rural areas of the developing countries,

they continue to be used as the primary source of medicine (Saleh*et al.*, 2015). About 80% of the people in developing countries use traditional medicines for their health care. The natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals. With respect to diseases caused by microorganisms, the increasing resistance in many common pathogens to currently used therapeutic agents, such as antibiotics and antiviral agents, has led to renewed interest in the discovery of novel anti-infective compounds.

As there are approximately 500000 plant species occurring worldwide, of which only 1% has been phytochemical investigated, there is great potential for discovering novel bioactive compounds. There have been numerous reports of the use of traditional plants and natural products for the treatment of oral diseases. Many plant-derived medicines used in traditional medicinal systems have been recorded in pharmacopeias as agents used to treat infections and a number of these have been recently investigated for their efficacy against oral microbial pathogens. The general antimicrobial activities of medicinal plants and plant products, such as essential oils, have been reviewed previously (Zomorodian, Kamiar, 2015).

Ayurveda is a medical system primarily practiced in India that has been known for nearly 5000 years. It includes diet and herbal remedies, while emphasizing the body, mind and spirit in disease prevention and treatment (Morgan, 2002). The term comes from the Sanskrit root Au (life) and Veda (knowledge). As the name implies it is not only the science of treatment of the ill but covers the whole gamut of happy human life involving the physical, metaphysical and the spiritual aspects. Ayurveda is gaining prominence as the natural system of health care all over the world.

Traditional medicine is commercialized and exported in a variety of settings. Some TM holders have chosen to market their knowledge outside of traditional settings. China, for example, promotes global TCM use to foster domestic economic development. Exports of TCM products from China generate billions of U.S. dollars in revenue annually. China's situation is not unique. In 2004, China accounted for only five percent of the global market for TM (Wu, Bingfang, 2014).

Plant essential oils (EOs) are produced predominantly using steam distillation, but can also be generated using fermentation, crushing, extraction, hydrolysis, and airing (Katarzyna *et al.,* 2019). EOs are used extensively in cosmetics in many different forms as perfumes, in antiseptic applications, and in domestic cleaning products (Stringaro *et al.,* 2018). They are volatile liquids or semi-liquids that are limpid, but are rarely colored, and are soluble in organic solvents. All of the parts of the plants can synthesize EOs, which are stored in secretary compartments such as cavities, canals, epidermic cells, or glandular trichomes. EOs is complex mixtures of terpenoides containing sesquiterpene and monoterpenes, and their oxygenated derivatives. EOs may also incorporate a variety of other molecules such as fatty acids, oxides, and sulfur derivatives (Falcão *et al.,* 2015). Both the terpenoid and phenylpropanoid families, which are sometimes identified as the principal constituents of several EOs, can constitute 85% of the total concentration of the oil. There are about 3000 well-recognized EOs, of which 300 are widely sold (Stringaro *et al.,* 2018).

2.4 Pharmacological activity of antioxidants

Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reaction can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves. Antioxidants from spices and herbs have the potential for large scale applications. Spices have been used not only for their flavoring properties but also for their food preserving abilities. Over the past few years a number of medicinal plants have been investigated for their quenching activity of specific ROS (Karnan and Subramani, 2015).

Antioxidants can act by diverse mechanisms in the oxidative sequence. The human body complex antioxidant defense system consists of the dietary intake of antioxidants, as well as the endogenous production of anti-oxidative compounds, such as glutathione (Long *et al.,* 2017). Antioxidant responses of our body can accommodate increased oxidative damage in diseased states to a level, but beyond those additional antioxidants are required to combat the increased stress.

Antioxidants may work either alone, or in association with each other against different types of free radicals. Vitamin E inhibits the propagation of lipid per oxidation; the combination of vitamin C and vitamin E suppress the formation of hydrogen peroxide; metal complexing antioxidant such as penicillamine inhibits free radical formation in lipid per oxidation (Kontoghiorghes *et al.,* 2019).

Oxidation is a metabolic process that leads to energy production necessary for essential cell activities. However metabolism of oxygen in living cells also leads to unavoidable production of oxygen derived free radicals, commonly known as Reactive Oxygen Species (ROS) (Venditti *et al.,* 2013). Adegoke, 2017 reported that ROS are involved in the onset of many diseases.

Natural antioxidants have been studied extensively for decades in order to find compounds protecting against a number of diseases related to oxidative stress and free radical - induced damages. This type of antioxidants occur in all higher plants and in all parts of the plants such as wood, bark, stems, pods, leaves, fruits, roots, flowers, pollen and seeds. *In vitro* experiments demonstrated that natural antioxidants are similar to the synthetic antioxidants of related structure (Yogesh *et al.,* 2015) and human consumption of antioxidants has many health benefits such as, prevention of oxidative damage associated with free radical damage and their contribution to disease such as cancer, the etiology of aging, coronary heart disease, ischemia-reperfusion injury, multiple sclerosis, Parkinson's disease, dementia, and autoimmune disorders (Vinay *et al.,* 2015).

These free radicals attack the unsaturated fatty acids of bio membranes, which results in lipid per oxidation and destruction of proteins and DNA, which causes a series of deteriorative change in the biological system leading to cell inactivation. Aziz *et al.,* 2019 highlighted that the ROS such as superoxide anion (O_2-), 20 hydroxyl radical (OH), singlet oxygen (O_2), H_2 O_2 and reactive nitrogen species (RNS) such as NO- , NO_2- and NO_3.

Free radicals generated from the oxygen are called Reactive Oxygen Species, which cause damage to other molecules by extracting electrons from them in order to attain stability. ROS are ions, atoms or molecules that have the ability to oxidize the reduced molecules. The most important reactive oxygen species (ROS) are the superoxide anion radical ($O_2^{\cdot -}$), hydrogen

peroxide (H_2O_2), alkoxyl ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$), hydroxyl radicals ($OH^{\cdot}$), and hypochlorous acid (HOCl). Other non-oxygen species existing are reactive nitrogen species (RNS), such as nitric oxide ($NO^{\cdot}$) and peroxynitrite (Sukri and Nursyuhada Mohd, 2017).

Hepel *et al.,* 2015 stated that oxidative stress that generates oxygen derived species is one of the major causes of many chronic and degenerative diseases including cancer. Pourahmad, 2016 suggested that since free radicals are involved in the both initiation and promotion stage of carcinogenesis, the free radical scavengers should function as inhibitors in neoplastic processes. Baldissera *et al.,* 2016 suggested that the most effective path to eliminate and diminish the action of free radicals is antioxidant defense mechanism.

2.5 *M.tinctoria* Roxb. –An overview

Morinda tinctoria Roxb. Commonly called as Nuna or manganatti belonging to Rubiaceae, is a moderate sized deciduous tree with spongy deeply cracked yellowish bark cultivated throughout the hotter parts of India. The leaves are 15-25cmlong, oblongtolanceolate. The flowers are tubular, white, scented, about2cmlong.The fruit is a green syncarp,2-2.5cmdiameter. Morinda are distributed throughout Tamilnadu and Kerala. However, the species *M.tinctoria* is present abundantly in most parts of Tamilnadu and in some parts of Kerala. The leaves of *Morinda tinctoria* Roxb. Are traditionally used to cure diarrhoea, ulcerative stomatitis, gastropathy, wounds, gout, hernia and fever (Longmann, 1997). The leaves are reported to possess antimicrobial activity (Jayasinghe *et al*., 2002).The major components have been identified in the Nuna plant which includes octoanicacid, potassium, vitamin C, terpenoids, scopoletin, flavones, glycosides, linoleic acid, anthraquinones, morindone, rubiadin and alizarin (Duduku *et al*., 2007).

2.6Pharmacognostic Study

Disease, treatment and the drugs have to be considered together in any medical systems. The secret of their medicinal properties lies in the active principles inherent in them. Since the early days of mankind plants with secondary metabolites have been used by humans to treat infections, health disorders and illness (Wink *et al*.,2005; Mann, 1992; Roberts and Wink, 1998 and Van and Wink, 2004) Thus, in order to increase the efficacy of the drugs in medicine, the use of plants for various diseases in Indigenous systems of medicine, identification of the active metabolites and their properties require investigation. Many phytochemical studies have been

carried out for thousands of medicinal plants belonging to various families of plants. Rubiaceae forms one of the important families consisting of numerous plants that have medicinal value but pharmacognostic study has not been done so far. Thus, detailed pharmacognostic studies on some of the important medicinal plants of the family Rubiaceae have been carried out. The taxes of Rubiaceae have certain diagnostic characteristic features. Some taxes are trees *Morinda tinctoria* Roxb..The leaves are opposite or whorled and entire with interpetiolar or interpetiolar foliaceous stipules. Flowers are usually decussate borne in panicles or cymes, sometimes aggregated into heads. The flowers are bisexual usually actinomorphic, sometimes zygomorphic showing bilabiate characters. The calyx is usually with 5 sepals, sometimes 4 and gamosepalous. 24 Corolla is tetramerous or pentamerous, tubular, rotate or funnel-shaped and the aestivation may be valvate, imbricate or contorted. Androecium has 4-5 stamens, inserted and alternate with the corolla lobes and epipetalous. Anthers are indorsed, two-celled and with longitudinal dehiscence. Ovary is usually two celled with a fleshy disc and inferior. One or more ovules are found in each cell and style is filiform, sometimes bifid or multifid. Fruit is usually a capsule, berry or drupe. Seeds are endospermic, sometimes winged (Rendle, 1979).Pharmacognostic Evaluation of *M. tinctoria.* It is documented that 80% of the world population has faith in traditional medicine particularly plant drug for their primary health care (Dubey *et al.,* 2004).

The number of reports of patients experiencing negative health consequences caused by the use of herbal medicine has increased in recent year. Analysis and studies have revealed a variety of reasons for such problem. One of the major causes of reported adverse events are directly linked to the poor quality of herbal drug, including raw medicinal plant materials (Ricardo, 2006).Thus, the traditional medicine required intensive and urgent investigation in the next few years from a botanical, chemical, and biological perspective, particularly for the diseases of the developing world and validated and standardized traditional medicinal agents must become a critical component for sustainable global health care (Geoffrey, 2003).

Recently, many international authorities and agencies, including the World Health Organization, European Agency for the Evaluation of Medicinal Products and European Scientific Cooperation of Phytomedicine, US Agency for Health Care Policy and Research, European Pharmacopoeia Commission, Department of Indian System of Medicine have started

creating new mechanisms to induce and regulate quality control and standardization of botanical medicine. For Ayurveda medicine and other traditional medicines, newer guidelines of standardization are required. A botanical drug or a preparation thereof is now regarded as one active substance in its entreaty, whether or not the constituents with therapeutic activity are known. This will be a major step in the development of new generation standardized botanical medicines. The WHO has published official documents on medicinal plants and WHO monographs on selected medicinal plants. (Bhushan *et al*, 2004).

KINGDOM	Plantae
CLASS	Tracheophytes
CLASS	Angiosperms
CLASS	Eudicots
CLASS	Asterids
ORDER	Gentianles
FAMILY	Rubiaceae
GENUS	*Morinda*
SPECIES	*M.tinctoria*

M. tinctoria Roxb. is a moderate to large size evergreen tree, generally about 15 m high. Stem is ash coloured dark, fissured with densely spreading crown. Leaves are 6.3-10 cm long and 3.2-5 cm broad pointed with serpentile ends or elliptic or obovate and leathery with wavy margins with petioles 1.3-2.5 cm long. Petioles and twigs produce watery milky exudates. Calyx is 1 cm long, fulvous pubescent. Corolla white coloured, sweet in fragrance, corolla longer than calyx, tube 1.5 mm long, lobes 8 mm long and are twenty four in numbers in two series; filaments short, glaborous, anthers glaborous, slightly twisted, acuminate; staminodes eight in number alternate with the stamens. Ovary silky pubescent; style grooved, slightly longer than corolla. Berry about 2.5 cm long, ovoid, yellow when ripe, contains a juicy pulp. Seeds are solitary, ovoid, compressed, brown and shining. *M. tinctoria* Roxb. is native in India.

2.6.1 Secondary metabolites

The use of plant drugs for medical treatment is possible since plants have evolved bioactive secondary metabolites that have been selected during evolution as a means against, for example, microbes and herbivores (Wink *et al.*, 2005;Harbome, 1993 &Seigler, 1998).On a global scale, medicinal plants are mainly used as crude drugs and their extracts (Van and Wyk, 2004). As the medicinal properties of herbal drugs are due to the presence of secondary metabolites detailed literature survey of some of the secondary metabolites such as alkaloids, saponins, phenols and tannins was carried out regarding their properties and their uses in the treatment of diseases in the Indigenous Systems of Medicine. Plants are the natural chemical factories for both primary and secondary metabolites. Secondary metabolites are chemical compounds in plants that are not involved in the normal growth, development or reproduction of plants and their absence may not affect the plants much like the primary metabolites. The secondary compounds are produced in the plants for various functions, such as defense against predators, parasites and diseases, for interspecific competition and to facilitate reproduction through colour, scent, etc. From pharmacological viewpoint secondary metabolites like alkaloids, glycosides, flavonoids, steroids, saponins, tannins and phenols are important. The type and the amount of the compounds may vary from species to species. Hence, extensive review has been done on the secondary metabolites stressing on their medicinal properties, their use for treatment of diseases and screening of their presence in the plants of study. Such studies will ensure that the plant resources are used economically and also increase the efficacy of the drugs in the treatment of diseases.

2.6.2Alkaloids

The term alkaloid was derived from the word "alkali-like" and was introduced by Meissner, a German pharmacist in 1819. Alkaloids are derivatives of amino acids and are bases containing one or more nitrogen atoms with relatively low molecular mass and are often found in a ring structure. 26 Many alkaloids like strychnine or coniine are poisonous, while some are used in medicine as analgesics and anesthetics like morphine and codeine. Gokhale (1979) stated that they are mainly produced by plants for protection against predators, detoxification of harmful chemicals, regulation of growth, reproduction and metabolism and as reservoirs for protein synthesis. They have a bitter taste. Michel *et al.* (1999) evaluated in vitro antimalarial activities

of 46 alkaloids. Two types of quasidemeric alkaloids were found to exhibit high and selective activities against Plasmodium spp. causing fever. Druilhe *et al. (1988*) studied on the activity of alkaloids like quinine, quinidine, cinchonine and cinchonidine from Cinchona bark on Plasmodium falciparum in vitro showed varied effects of the alkaloids. Foumet *et al*. (1993).

2.6.3Saponins

Saponins are glycosides of steroids, steroid alkaloids or triterpenes that form a waxy protective layer on parts of the plant surface. Saponins are glycosides which usually, contain sapogenin as an aglycone part and have soap like action. According to Kokate *et al.,* (1999) steroidal saponins consist of teracycle triterpenoid and pentacyclic triterpenoid saponins. They form foam with water to give colloidal solutions and they are heamotoxic. They have a bitter and acrid taste and irritate mucous membrane. They are crystalline 27 substances soluble in water and alcohol but insoluble in non-poplar organic solvents.

2.6.4 Phenols

Harbome (1984) describes phenols as compounds containing an aromatic hydrocarbon ring bearing one or more hydroxyl (-0H) substituents. The simplest molecular formula of the group is phenol (CeHsOH). They usually occur in combination with sugar as glycoside in cell vacuoles. According to trease and Evans (2002) phenols constitute the largest group of plant secondary metabolites. Many organic compounds like tannins, coumarins and their glycosides, anthraquinones and their glycosides, naphthoquinones, flavones and flavonoids glycosides, anthocyanidins and anthocyanins, lignans and lignin are different classes of phenols. They are soluble in water and adverse effects on insect feeding. Vattem *et al*. (2005), Catherine (2007) and Puupponen- Pimia *et al*., (2001) studies on cranberry found phenolic compounds to have health benefits and antimicrobial properties. The use of salicylic acid and trichlorophenol in medicine for its analgesic, antipyretic and anti-inflammatory actions and the latter as an antiseptic has been mentioned.

2.6.5 Tannins

Tannins are compounds present in plant extracts that combine with proteins of animal skin to form leather and the term 'tannin' was first used by Seguin in 1796. Van-Burden and

Robinson (1981) reported the formation of complexes between protein and tannins. According to Harbome (1984) tannins are associated with woody tissues of angiosperms. Two types of tannins are known viz. condensed tannins generally found in the ferns, gymnosperms and woody angiosperms. Condensed tannins or proanthocyanidins are not hydrolyzed easily and do not contain a sugar moiety. While the second type, the hydrolysable tannins can be hydrolyzed by acids or enzymes and have a central sugar moiety and are found in a few families of dicotyledonous plants. Gallic acid is hydrolysable tannin. Ansari (2006) mentions their presence in leaves, fruits, barks or stem, generally occurs in immature fruits but disappears.

2.7 Phytochemical constituents – *M.tinctoria*Roxb.

2.7.1 Stem bark

Taraxerone, taraxerol, betulinic acid and spinasterol, sodium salt of betulinic acid and ursolic acid, fatty acid esters of alpha-spinasterol, farnan-2-one-3 betaol farnan3-one, and olean-18-en-2-one-3-ol and lup-20 (29)- en-3 beta-ol, triterpene 3β-hydroxy-lup-20(29)-ene23, 28-dioic acid, beta amyrin, lupeol, alpha cadinol, taumuurolol, hexadecanoic acid, diisobutyl phthalate, octadecadienoic acid, new gallic acid esters, (phenyl propyl gallate) are important molecules obtained from stem bark. The tree also yields a gum. Bark also contains tannins, wax, coloring matter and starch.

2.7.2 Fruit and seed

Fruit and seed showed presence of quercitol, ursolic acid, dihydroquercetin, quercetin, β-d glycosides of beta sitosterol, alpha spinasterol, acid and M.tinctoria, pentacyclictriterpenes 3beta, 6beta, 19alpha,23-tetrahydroxy-urs-12-ene and 1betahydroxy-3beta-hexanoyllup-20 (29)-ene-23, 28- dioic acid, *M.tinctoria*, mi-saponin A 16 alpha-hydroxy mi-saponin A, taxifolin, alphaspinasterolglucoside, Miglycoside 1, mimusopside A and B.

2.7.3 Leaves heartwood and roots

Leaves contain hentriacontane, carotene and lupeol. A new steroidal saponin, 5 alpha-stigmast-9(11) en-3- obeta- D - glucopyranosyl (1-5) - o-beta-dxylofuranoside was isolated from the roots of *M.tinctoria* Leaves contain qurecitol, lupeol, -sitoserol,β-carotene, d-mannitol, βhentriacontane, -d glucoside and qurecetin. Theβ-sitosterol-β lipid concentration of leaves was

higher in summer (32.7 mg/gm) over that of monsoon (29.75 mg/gm) and winter (30.7 mg/gm). The bark of lipid concentration was ranging from 13.5 to 16.8 mg/gm) summer (16.8 mg/gm) show highest content over other season i.e. monsoon (13.5mg/gm) and winter (14.7 mg/gm).

2.7.4 Seeds

The seed kernels yield 16-25% of a fatty oil qurecitol, dihydroqurecitin, qurecitin, ursolic acid, b sitisterol glycosides. The fat free seed meal yield 2.4% bassic acid (C30H46O5), a characteristic sapogenin of sapotaceae. It also yields a saponin which on hydrolysis yields ramnose (2 mol.), arabinose(2 mol.) and glucose(1 mol.).

Preliminary phytochemical screening revealed the presence of phytochemical constituents such as terpenoids, saponins, anthraquinone glycoside and cardiac glycoside. isolated two new pentacyclictriterpenes, and from its seeds and characterized as 2β,3β,23-trihydroxy-28-noroleana-5, 12-dien-16-one and 3β, 23- dihydroxyoleana - 5, 12 – dien – 16 - one, respectively, based on their spectroscopic properties. Sahu *et al*., isolated a novel minor triterpenoid saponins mimusin {3-O-[β-d-glucopyranosyl- (1→6)-β-d glucopyranosyl]-2β,3β,6β,23-tetrahy droxyolean-12-en-28-oic acid 28-O-α-l-rhamnopyranosyl - (1→3) – β - d-xylopyranosyl - (1→4) – α - l rhamnopyranosyl - (1→2) – α -l-arabinopyranoside} from the seeds, in addition to two known triterpenoidsaponins, Mi-saponin A and 16α-hydroxyMi-saponin A. The structure of the minor saponin was established by comparing its 1 3 C NMR and LS-MS linked-scan, ESI-MS data with FAB-MS of the mimusopsin isolated earlier from the same source.

2.7.5 Flower

The flowers are tubular, white, scented, about 2 cm long.Fresh flower contain 2-Phenylethanol, 4- hydroxyl benzene methanol and cinnamyl alcohol, whereas dried flowers contain long chain carboxylic acid ester and (Z)-9-octadecenoic acid. Jahan *et al*., isolated a new triterpene 3β-hydroxy-lup-20(29)-ene-23,28- dioic acid. Bhuyan and Saikai extracted dye from *M.tinctoria* 20 (Akhtar *et al*.,2020) isolated a new farnane-type triterpenoid, farnan-2-one-3β-ol, from the stem bark of *M. tinctoria.*

2.8Molecular docking studies

Molecular docking techniques aim to predict the best matching binding mode of a ligand to a macromolecular partner (here just proteins are considered). It consists in the generation of a number of possible conformations/orientations, i.e., poses, of the ligand within the protein binding site. For this reason, the availability of the three-dimensional structure of the molecular target is a necessary condition; it can be an experimentally solved structure (such as by X-ray crystallography or NMR) or a structure obtained by computational techniques (such as homology modeling) (Salmaso, 2018).

Molecular docking is composed mainly by two stages: an engine for conformations/orientations sampling and a scoring function, which associates a score to each predicted pose (Salmaso *et al.*, 2018). The sampling process should effectively search the conformational space described by the free energy landscape, where energy, in docking, is approximated by the scoring function. The scoring function should be able to associate the native bound-conformation to the global minimum of the energy hyper surface.

Computer Aided Drug Design (CADD) techniques are used principally for three reasons: virtual screening hit/lead optimization and design of novel compounds. In virtual screening a huge database of compounds is examined searching for binding capacity for a target and a subset of compounds is picked out and suggested for *in vitro* testing; the purpose is to increase the hit rate of novel drugs by reducing the number of compounds to test experimentally. The second application of CADD is the optimization of a hit/lead compound driven by the rationalization of a structure-activity relationship. After the individuation of key elements for binding, the design of new compounds can be attempted (Salmaso, 2018).

CADD methods may be classified as ligand-based (LB) and structure-based (SB), depending on the availability and employment of the target structure (Sliwoski *et al.*, 2014). In the framework of CADD, structure-based drug design (SBDD) methods take advantage of the abundance of experimentally solved structures in the Protein Data Bank (Salmaso *et al.*, 2018), which can possibly be used also as templates for homology models if the structure of interest is lacking. SBDD is based on the premise that the knowledge of the target structure can help to rationalize and optimize binding since ligand-target interactions are mediated by their

complementarily. With the evolution of the binding models, it is clear that speaking of "target structure" is an approximation, given that proteins fluctuate among an ensemble of structures (Salmaso *et al.,* 2018).

In this post-genomic era, research increasingly focuses on proteomics. Experimental and computational efforts are devoted to large-scale generation and analysis of information derived from 3D structures and dynamics of proteins, with the goal of scientific and commercial break-through in drug discovery (Raghavendra *et al.,* 2015).

In silico drug design can play a significant role in all stages of drug development from the preclinical discovery stage to late stage clinical development. Its use in the drug development helps in selecting only a potent lead molecule and may thus prevent the late stage clinical failures; thereby a significant reduction in cost can be achieved (Yuliana *et al.,* 2013) Screening of pharmacological activity of active ingredients in medicinal plants is an expensive process, requiring energy, qualified human resources and require a long time if done in laboratory experiments using experimental animals (Atanasov and Atanas, 2015).

Molecular docking is a method which used to predict an inter molecular complex between the drug molecule with its target protein. When performing molecular docking, a set of data which contains information on the ligand or drug to be docked and protein targets to be used are needed. Elokely *et al.,* 2013 reported that the information required in this process include three-dimensional structure of the ligand and target protein.

Molecular docking, performed through computer programs like MOE, is very helpful in studying the interactions of ligand molecules with the target protein before its *in vitro* synthesis (Abrigach and Farid, 2018). Docking is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets which in turn predict the affinity and activity of the small molecule.

Hence docking plays an important role in the rational design of drugs (Maggio and Julián, 2021). Given the biological and pharmaceutical significance of molecular docking, considerable efforts have been directed towards improving the methods used to predict docking. Chen *et al.,* 2013 opined that the CADD is an *in silico* simulation technique used in the screening of compounds based on the structure and biological activity.

In silico is an expression used to mean "performed on computer or via computer simulation". This approach differs from use of expensive high throughput screening (HTS) in robotic labs to physically test thousands of diverse compounds a day often with an expected hit rate on the order of 1 per cent or less with still fewer expected to be real leads following further testing (Selvamani *et al*., 2016).

Docking is a computational approach which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex (Kitchen *et al*., 2004). Shruthila *et al*., 2014 had done docking studies with the MCFAs of coconut oil against Alzheimer's. The result indicated that these fatty acids bind with Amyloid beta plaque and inhibits its formation. Amyloid beta plaques are the main cause for Alzheimer's disease. Mala *et al*., 2015 conducted an *in silico* analysis using the tool Discovery Studio 4 to identify the bioactive compounds to target anti-apoptotic proteins- Bcl 2 and Bcl xl. Discovery Studio is a suite of software for simulating small molecule and macromolecule systems. It is developed and distributed by Accelrys, USA.

There are many types of use of this information obtained about chemical compounds. This will enable selection of compounds for probing target pathways, finding side effect of old compound or suggest new compounds, assessment of ADMET (absorption, distribution, metabolism, excretion, toxicity) properties of drugs, quantification of structure activity relationships for small compounds. This information can be used for defining many new pathway models which can be used for prediction works. Using the Molinspiration server (http://www. molinspiration.com/) molecular properties and drug likeness of the compounds can be examined on the basis of "Lipinski's Rule of Five" (Ahmed and Shimaa, 2020).

The Lipinski's rule, formulated by Christopher A. Lipinski in 1997 is a rule of thumb to evaluate drug likeness which states that an orally active drug has no more than one violation of following criteria i.e., has not more than 5 hydrogen bond donors, not more than 10 hydrogen bond acceptors, molecular weight below 500 Daltons, partition co-efficient log P less than 5 (Lipinski *et al.,* 2001).

Molecular docking is a method which used to predict an intermolecular complex between the drug molecules with its target protein. When performing molecular docking, a set of data which contains information on the ligand or drug to be docked and protein targets to be used

are needed. Elokely *et al.,* 2013 reported that the information required in this process include a three-dimensional structure of the ligand and target protein. Molecular docking, performed through computer programs like MOE (Molecular Operating Environment), is very helpful in studying the interactions of ligand molecules with the target protein before its*in vitro* synthesis (Abrigach and Farid, 2018).

Docking is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to predict the affinity and activity of the small molecule. Recently, docking ligands to receptors utilizing rational drug design is on the increase owing to a few problems in the conventional methods of drug designing. Hence docking plays an important role in the rational design of drugs (Adeniyi *et al.,* 2016). Given the biological and pharmaceutical significance of molecular docking, considerable efforts have been directed towards improving the methods used to predict docking.

3

MATERIALS AND METHODS

The materials used and methods adopted in the present study entitled**"Assessment of liposome encapsulated *Morinda tinctoria Roxb.* leaves for Wound healing activity**" is furnished below.

Fig.1. *M. Tinctoria* Roxb. plant and powdered sample

3.1 Preliminary phytochemical analysis

3.1.1Collection of the plant

Healthy leaves of *M.tinctoria* were collected from Coimbatore, India.

3.1.2 Preparation of leaf powder

The *M. tinctoria* were washed completely and let to dry for 5-7 days at ambient temperature. The dried-out leaves were ground to powder and stored in screw cap bottles until further analysis.

3.1.3 Preparation of the extract

50g of sample was dissolved in 50ml of various solvents (Petroleum benzene, Ethyl acetate, Ethanol, and chloroform). They were intermittently shaken with an electric shaker. It was then filtered and further concentrated by evaporation.

3.2 Qualitative phytochemical analysis

All the extracts were subjected to different phytochemical tests to determine the phytochemical constituents present in the extract (Raman, 2006). Various tests to determine the presence of alkaloids, flavonoids, sterols and triterpenoid, carbohydrates, tannins, proteins and amino acids, saponins, phenols and glycosides were performed as per the following procedure (Kokate*et al.*, 2008).

3.2.1 Test for alkaloids

- **Mayer's test:** A small quantity of solvent free methanolic extract was treated with Mayer's reagent (mercuric chloride and potassium iodide) and observed for yellowish buff color precipitate.
- **Dragendroff's test:** A small quantity of solvent free methanolic extract was treated with Dragendroff's reagent (sodium iodide, basic bismuth carbonate, glacial acetic acid and ethyl acetate) and observed for the presence of orange brown precipitate.
- **Wagner's test:** A small quantity of solvent free methanolic extract was treated with Wagner's reagent and observed for reddish brown precipitate.

3.2.2Test for flavonoids

- **Ferric chloride test:** A small quantity of the extract was added to a few drops of neutral ferric chloride solution and noted for the development of intense green color.

3.2.3 Test for sterols and triterpenoid

- **Libermann-burchard test:** 5 ml of test solution was boiled with two drops of acetic anhydride boiled and cooled then concentrated sulphuric acid was added along the side of the test tube. Appearance of brown ring at the junction of two layers is taken as reference. If the upper layer turns green, sterols are present whereas formation of deep red colour indicates the presence of triterpenoids.

- **Salkowski's test:** Test solution was treated with a few drops of concentrated sulphuric acid and shaken well and the solution was allowed to stand for some time. Appearance of red colour in the lower layer indicates the presence of sterols whereas formation of yellow colour in the lower layer indicates the presence of triterpenoid.

3.2.4. Test for carbohydrates

- **Fehling's test:** A few drops of test solution were boiled with equal volume of Fehling's solution. Formation of brick red precipitate confirmed the presence of reducing sugars.

3.2.5. Test for tannins

- **Ferric chloride test:** To 5 ml test solution, a few drops of 5% ferric chloride solution was added. Appearance of intense green or blue colour indicates presence of tannins.

3.2.6. Test for proteins

- **Biuret test:** The extract was treated with equal volume of 40% sodium hydroxide and 2 drops of 1% copper sulphate solution. Pink or purple colour indicated the presence of proteins.
- **Warming test:** Test solution was boiled in a boiling water bath. Appearance of coagulation indicated the presence of proteins.

3.2.7. Test for amino acids

- **Ninhydrin test:** A small quantity of test solution was boiled with 5% solution of Ninhydrin. Appearance of violet color indicated the presence of free amino acids.

3.2.8 Test for saponins

- **Froth test:** To the extract, 20ml of distilled water was added and agitated on a graduated cylinder for 15 min. Persistence of characteristic honey comb froth at least 1cm in height for 30 minutes indicated the presence of saponins.

3.2.9. Test for phenols

- **Ferric chloride test:** 2ml of the extract was treated with 2ml of 5% ferric chloride solution and the formation of deep blue or black colour indicated the presence of phenols.

3.3. Antioxidant studies

3.3.1. Free radical scavenging activity

The radical scavenging activities of the different extracts were measured in vitro against a battery of radicals namely DPPH

3.3.2 DPPH free radical scavenging activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich Co., St. Louis, USA. The diluted working solutions of the test extracts were prepared in petroleum benzene. About 1ml of graded concentration (20, 30, 40, 50, 75, 100 µg/ml) of extracts were taken in different test tubes and assorted with 1ml of DPPH (0.1Mm in petroleum benzene) and shaken well. This solution was then incubated in room temperature for 30 minutes. The optical density was recorded at 517 nm using UV spectrophotometer. Corresponding blank sample was prepared. Mixture of 0.5ml petroleum benzene and 0.5ml DPPH solution was used as control. The absorbance change was compared with the standard Quercetin (20-100µg/ml) and was determined. The scavenging activity was then calculated using the formula:

Radical scavenging activity (%) = Abs (control)–Abs (sample)/Abs (control) x 100

Where, Abs (control) is the absorbance of DPPH radical with petroleum benzene and Abs (sample) is the absorbance of DPPH radical with a sample extract or standard.

3.4 Liposome Synthesis

Liposomes were synthesized following the methods of Anwar *et al* (2018) with minor modifications. 1 gm of Lipoid P 30 (Lecithin) was diluted with dichloromethane, while 1 gram of crude ethanolic extract was diluted with 90% ethanol. The dissolved phosphor lipid and ethanolic extract were then added to the conical flask. The dichloromethane was evaporated

using a magnetic stirrer at 37°C at a constant speed ranging from 25 to 150 rpm until an even formed thin film layer was achieved. The layer was then refrigerated for up to 24 hours. The thin coating was then hydrated with phosphate buffer pH 5.5 while shaking at 40°C. After forming the Phytosomes suspension, the Phytosomes formulation was developed using ultrasonication for 5 minutes.

3.5 Antimicrobial Activity

3.5.1. Test organisms

Gram positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Enterococccus faecalis,*) and gram negative bacteria (*Pseudomonas aeruginosa*) were used for antibacterial activity.

3.5.2. Procedure

The antimicrobial activity of the ethanolic extract of *M.tinctoria* Roxb.leaves was determined using agar well diffusion method. The antibacterial activity of extract was tested against Gram positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Enterococcus faecalis*), Gram negative bacteria (*Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa*). Four wells were made in sterile Nutrient agar plates using a sterile cork borer, 50µl of each bacterial culture were swabbed on the respective plates. 20µl of each extract was added in the respective wells separately. The plates were incubated at 37 0 C for 24 hours to measure the zone of inhibition of the test extracts against a standard antibiotic. Amoxicillin was used as positive control.

3.6. *Insilico* studies

Computer-based methods are becoming increasingly important and complementary to wet laboratory experiments in studying the structure and function of bimolecular. The integration of computational and experimental strategies has been of great value in the identification and development of novel promising compounds. Docking studies are used at different stages of drug discovery such as to predict a ligand-receptor interaction and also to rank the compounds based on the binding energies or fitness score. Molecular docking plays a significant role in structural based drug designing by predicting the binding orientation of small molecule drug

candidates to their known 3D structures of the protein targets. The compounds from the review of literature were selected for *in silico* docking valuation at particular active binding sites of the target proteins.

3.6.1. Evaluation of drug likeliness and toxicity prediction

Lipinski's rule of five was used to scrutinize the ligands for drug-like properties. This rule defines molecular properties significant for a drug's pharmacokinetics in the human body and provides the evidence concerning the exploitation of the ligands as a drug (Lipinski *et al.*, 1997). The rules are:

1. Molecular weight < 500 daltons,
2. Number of hydrogen bond donors <5,
3. Number of hydrogen bond acceptors < 10 and
4. Calculated water partition coefficient (LogP) < 5.
5. Molar refractivity should be between 40-130

The ligands passing the Lipinski properties were taken for docking studies.

3.6.2 ADMET Prediction

pkCSM (Predicting small molecule pharmacokinetic and toxicity properties using graph based signatures) is a method for predicting and optimizing pharmacokinetic properties and toxicity properties. It uses graph based signatures approach. Graph modeling is an intuitive and well established mathematical representation of chemical entities, from which different descriptors encompassing both molecule structure and chemistry can be extracted. An intuitive graph representation of a compound can be achieved by representing atoms as nodes and their covalent bonds as edges. pkCSM adapted the cut off scanning concept to develop a predictive model of ADMET properties for drug development. The performance of pkCSM software in the external validation dataset showed an accuracy of 83.8% in the mutagenicity test. There are several endpoints of pkCSM, i.e LD, Ames test, maximum daily dose and hepatotoxic.

3.6.3. Bio activity score

The bioactivity score of the selected ligands was calculated for a nuclear receptor, GProtein Coupled Receptor, ion channel, kinase, and protease. These were done by obtaining

SMILES notations of the chosen compounds from pubchem database and feeding them in the online Mol inspiration software version 2011.06 (www.molinspiration.com) (Zhao *et al.,* 2002).

3.6.4. Target proteins

The Protein Data Bank (PDB) is an important resource in divisions of structural biology such as structural genomics. A majority of scientific journals, and a few funding agencies, such as the NIH in the USA, now insistresearchersto deposit their structure data to the PDB (Porollo*et al.,* 2007). In the present study three- dimensional structure of diseased protein such as1R5K, 1X7B, 3POZ, 1AGW and were selected from the protein data bank.

3.6.5. Molecular docking

The molecular docking studies were performed to determine the chemical interactions of the phytoconstituents of plant *M. tinctoria* with targeted protein responsible for wound healing. Initially the proteins and ligands were prepared. All the hetero atoms were removed from crystal structure of protein to make all the complex receptors free of ligand before docking. AutoDockTools (version-4.2.6) software was used for the preparation and optimization of protein and ligand molecules. Water molecules were removed, and polar hydrogens and Kollman charges were added in the protein molecule. Since ligands were small molecules, Gasteiger charge was added. The binding site of the protein was selected by selecting a grid box with varying dimensional sizes and docked. The results were analyzed on the basis of binding affinity of ligand with the protein. Images of protein-ligand binding confirmations were visualized and processed using Discovery studio visualizes 2020 software.

4

RESULTS

The results pertaining to the study entitled "**Assessment of liposome encapsulated *Morinda tinctoria* Roxb. leaves for wound healing activity**" are presented under the following headings:

4.1 Qualitative phytochemical analysis

Qualitative analysis of *M.tinctoria* Roxb. was carried out to identify the presence of the major phytochemical. Results showed the presence of alkaloids, carbohydrates, flavonoids, tannins and phenols (Table I). Maximum intensity of the phytochemical was observed in ethanol extract.

Table I. Phytochemical Screening of *Morinda tinctoria* Roxb.leaves

S.No.	Chemical Constituent	Name of the test	Ethanol	Chloroform	Water	Petroleum benzene
1	Alkaloid	Mayer's test	++ -	- - -	+ ++	- - -
		Dragendrofs test	+++	- - -	+++	- - -
		Wagner's test	++-	- - -	- - -	- - -
2	Carbohydrates	Molischs test	- + -	- - -	- - -	- - -
		Benedicts test Fehling's test	- - -	- - -	- - -	- - +
3	Glycosides	Brontragers test	++-	+++	- - -	- - +
		Legal test	- - +	- - +	- - -	- - -
4	Saponins	Foam test	-++	- - -	- + -	+++
		Froth test	+++	- - -	- - -	- - -
5	Phytosterols	Leibermannburchard test	+ + -	- - -	+ + +	- - -
6	Phenols	Ferric chloride test	- + -	+ + +	+ + -	+ + +
7	Tannins	Alkaline reagent test	+ - +	- - -	- - -	+ + +
		Gelatin test	- ++	- - -	- - -	+ + +
8	Flavonoids	Lead acetate test	- - -	+- -	- - -	- - -
		Alkaline reagent test	- - -	- - -	- - -	+ + +
9	Proteins and amino acids	Ninhydrin test	- - +	- - -	+ - +	- - -
		Biuret test	+ + -	- - +	- - -	- - -
10	Diterpenes	Copper acetate test	- - -	- - -	- - -	- - -

\+ Presence of respective class compound
\- Absence of respective class compound

4.2 Antioxidant activity

4.2.1 Analysis of Radical scavenging activity using DPPH Assay

The radical scavenging activities of these extracts were determined *In vitro* against Ascorbic acid based on color change which is due to the reduction reaction. The highest scavenging efficacy of *M. tinctoria* Roxb.was 95.08%. The extent of DPPH scavenging by nanoparticle solution was significant, where the stable radical was effectively reduced to the yellow-colored compound di phenylpicryl hydrazine. The assay is based on the scavenging capacity of antioxidants towards a stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH). The ethanolic extract of *M.tinctoria* Roxb. leaf showed the highest scavenging activity with an absorbance value of 95.08% and 85.25% (Table II).

Table II. DPPH radical scavenging activity of*M.tinctoria*Roxb.

Samples	Concentration(µl)	% inhibition
Standard (Ascorbic acid)	30	68.85
	60	77.87
	90	86.89
	120	93.44
	150	97.54
Sample Ethanol	10	**95.08**
	50	**85.25**
	150	71.31
	250	56.56
	500	24.59

4.3 Antibacterial activity

Liposome loaded with *M. tinctoria* Roxb. leaf extract was evaluated for its antibacterial activity against six clinical bacterial isolates of Gram positive bacteria (*Staphylococcus aureus*, *Proteus vulgaris, Pseudomonas aeruginosa* and *Enterococcus faecalis*) and Gram negative bacteria (*Escherichia coli, Klebsiella pneumoniae*). Table III describes the antibacterial activity of Ampicillin, liposome of *M. tinctoria* Roxb. leaves against the selected bacterial isolates. Fig 11 shows specific activity of each extract in zone formation against each bacterial isolate. From the Table III, it was observed that the zone of inhibition of the synthesized liposome loaded with *M. tinctoria* Roxb. leaf extract exhibited the zone of inhibition of *Escherichia coli* (13 mm), *Proteus vulgaris* (14 mm), *Staphylococcus aureus* (13 mm), *Pseudomonas aeruginosa* (15 mm) *Enterococcus feacalis* (13 mm) and *Klebsiella pneumonia* (14 mm).

Table III. Antibacterial activity of *M. tinctoria*Roxb. leaves against the selected bacterial isolates

S.No	Name of the organism	Ampicilin (mm)	Ethanol (mm)	Liposome (mm)	Plant extract (mm)
1	*Pseudomonas aeruginosa*	11	6	15	13
2	*E-coli*	14	9	13	15
3	*Staphylococcus aureus*	17	20	13	14
4	*Proteus vulgaris*	15	5	14	24
5	*Klebsiella pneumonia*	NA	7	14	21
6	*Enterococcus faecalis*	12	21	13	19

4.4 *In silico* studies

4.4.1 Ligand retrieval

PubChem is a huge database for small molecule deposition from NMR and XRD derived structures. The structure of ten molecules which had CAS numbers was collected from the PubChem database.

Table IV. Ligands chosen for docking

Compound Name	Molecular formula	Molecular weight
Cynarin	$C_{25}H_{24}O_{12}$	516.4
Gallicacid	$C_7H_6O_5$	170.12
Kaempferol-3-O-rutinoside	$C_{27}H_{30}O_{15}$	594.5
Mangiferin	$C_{19}H_{18}O_{11}$	422.3
Oleuropein	$C_{25}H_{32}O_{13}$	540.5
Orientin	$C_{21}H_{20}O_{11}$	448.4
Quercitin	$C_{15}H_{10}O_7$	302.23
Rutin	$C_{27}H_{30}O_{16}$	610.5
Steviol	$C_{20}H_{30}O_3$	318.4
Ursolic acid	$C_{30}H_{48}O_3$	456.7

4.4.2 Drug likeliness and prediction of toxicity of the selected molecule

Using PubChem database, the drug-like characteristics of the 10 molecules were appraised. The rule defines a molecule as drug like only if all of the following conditions are satisfied:

- molar weight (MW) is less than five hundred Daltons (Da);
- The log of the “octanol/water partition coefficient” (QPlogPo/w) is less than five,
- The total of hydrogen bond acceptors (HBA) is less than10 and
- The total of hydrogen bond donors (HBD) is less than five.

These parameters were calculated and the drug like nature of the compounds of *M.tinctoria* Was tabulated in Table V.

Table V. Compliance of compounds to computational parameters of drug likeness (Lipinski's rule of five)

S. No	Compound	MW(g/mol)	XLOGP3	HBD	HBA	Rule of 5 violation
1	Cynarin	516.4	1.5	7	12	3
2	Gallicacid	170.12	0.7	4	5	0
3	Kaempferol-3-o-rutinoside	594.5	-0.9	9	15	3
4	Mangiferin	422.3	-0.4	8	11	2
5	Oleuropein	540.5	-0.4	6	13	3
6	Orientin	448.4	-0.2	8	11	2
7	Quercitin	302.23	1.5	5	7	0
8	Rutin	610.5	-1.3	10	16	3
9	Steviol	318.4	3.8	2	3	0
10	Ursolicacid	456.7	7.3	2	3	1

4.4.3 Bioactivity score

The bioactivity score of the selected compounds present in *M.tinctoria* was calculated for ion channel modulator, nuclear receptor ligand, GPCR (G-Protein Coupled Receptor) ligand, kinase inhibitor, protease inhibitor and enzyme inhibitors. These properties serve as an indication of excellent pharmacological activities *In vivo*.

Table VI. Bioactivity scores of the selected ligands

S.No	Compound Name	GPCR Receptor	Ion channel receptor	Protein kinase receptor	Nuclear receptor	Protease receptor	Enzyme inhibitor
1	Cynarin	0.18	0.04	-0.01	0.50	0.21	0.42
2	Gallicacid	-0.77	-0.26	-0.88	-0.52	-0.94	-0.17
3	Kaempferol-3-0 rutinoside	-0.01	-0.43	-0.09	-0.17	-0.04	0.18
4	Mangiferin	0.06	-0.04	0.06	0.14	-0.03	0.48
5	Oleuropein	0.22	0.14	-0.22	0.08	0.11	0.31

6	Orientin	0.12	-0.14	0.20	0.20	0.01	0.45
7	Quercitin	-0.06	-0.19	0.28	0.36	-0.25	0.28
8	Rutin	-0.05	-0.52	-0.14	-0.23	-0.07	0.12
9	Steviol	0.40	0.18	-0.28	0.73	0.14	0.52
10	Ursolic acid	0.28	-0.03	-0.50	0.89	0.23	0.69

The bioactivity score of ligands which were chosen from review of literature of *M. tinctoria* Roxb. leaves were calculated for GPCR (G-Protein Coupled Receptor) ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor and enzyme inhibitors. The bioactive scores fall on the following ranges such as active > 0, moderately active > −5.0 to 0.0 and inactive <-5.05. The bioactivity analysis revealed that all the compounds were moderately bioactive (Table VI).

4.4.4. Diseased Protein for Wound healing

The selection of target protein for wound was made through literature survey. There are four proteins retrieved from PDB. Accordingly, the protein structures were retrieved on basis of a number of criteria such as single monomer, less than 2 resolution, Homo sapiens and single domain in the protein complex. All ligands chosen from *M.tinctoria* Roxb. leaves were docked against the four chosen wound proteins such as 1R5K, 1X7B, 3POZ and 1AGW. The structure of the wound proteins derived from PDB is shown table VII.

Table VII. Target wound proteins with their characteristics and structure

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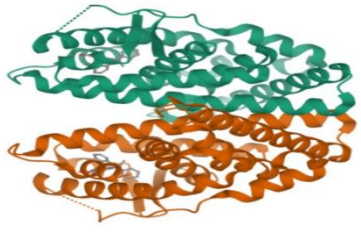
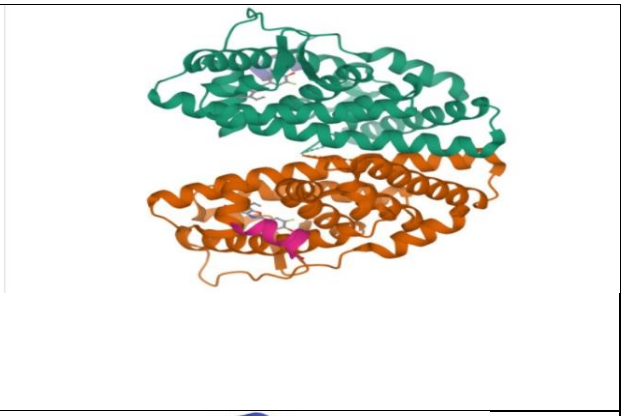
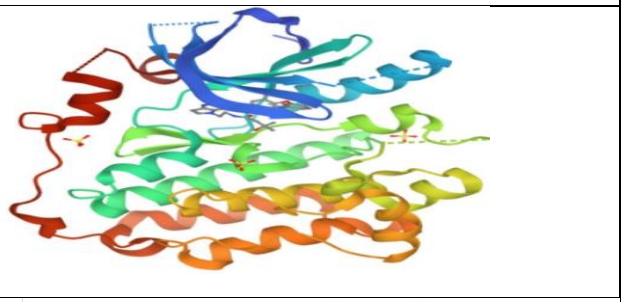
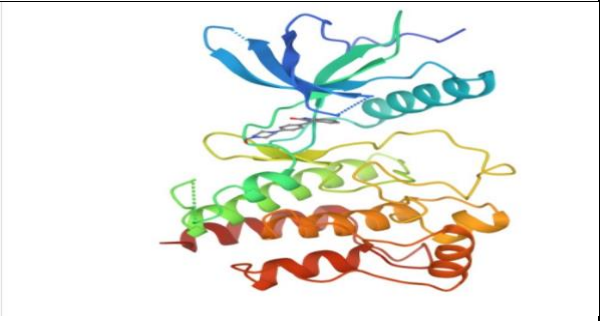
Name of the protein	PDB code	Role of the protein	3D structure of the protein
Human Estrogen Receptor alpha Ligand-Binding Domain In Complex With GW5638	1R5K	Structural basis for an unexpected mode of SERM-mediated ER antagonism.	

Crystal structure of estrogen receptor beta complexed with ERB-041	1X7B	Protein synthesis is the creation of proteins that allow the function and structure of cells, based on the genetic information of DNA	
EGFR Kinase domain complexed with tak-285	3POZ	The process of protein synthesis serves as a method to produce proteins for the body.	
Crystal structure of the tyrosine kinase domain of fibroblast growth factor receptor 1i complex with SU4984 inhibitor	1AGW	Proteins are molecules that help the body to do work, or in some cases, to express genes that are encoded in DNA.	

4.4.5 Molocular docking

The results showed that Steviol and Ursolic acid exhibited a significant docking interaction with wound protein, 1R5K with the docking score of -6.56 and -5.11kcal/mol (Table VIII). Quercetin, Steviol exhibited a significant docking interaction with wound protein, 1X7B, with docking scores of-7.23 and 7.06 kcal/mol showing good binding energy. Gallic acid and Ursolic acid exhibited a significant docking interaction with wound protein 3POZ with docking scores of -5.34 and -6.63 kcal/mol. Steviol and ursolic acid exhibited a significant docking interaction with wound protein, 1AGW, with docking scores of -6.81 and -6.15kcal/mol.

The inference of the binding score indicated that the unique molecule from *M. tinctoria* Roxb. which showed good binding affinity with each diseased protein. So, it can act as wound healing drug for the analyzed wound healing proteins. Hydrogen bond energy, and residual interaction are the parameters considered for the calculation of the results.These parameters enable the determination of the binding affinity of ligand with the protein. The quality of the ligand's binding mode to the protein is directly proportionate to the number of hydrogen bonds in the structure. Location of the ligand's bond to amino acid of the protein is shown by the Residual interaction.

Table VIII. Summary of docking score of the top ranked poses in each protein

Protein	Compound name	Binding score(kcal/mol)
1R5K	Kaempferol-3-0-rutinoside	-1.13
	Mangiferin	-4.22
	Oleuroein	-3.36
	Orientin	-3.2
	Quercetin	-4.64
	Rutin	-0.95
	Steviol	**-6.56**
	Ursolic acid	**-5.11**
	Gallic acid	-2.58
	Cynarin	-0.93
1X7B	Cynarin	-3.89
	Gallic acid	**-5.02**
	Kaempferol-3-0-rutinoside	-1.4
	Mangiferin Oleuropein	-3.7 -2.43
	Orientin	-1.59
	Quercetin	**-7.06**
	Rutin	-2.0
	Steviol	**-7.23**
	Ursolic acid	**-6.65**
3POZ	Gallic acid	**-5.34**
	Kaempferol-3-0-rutinoside	-2.9

	Mangiferin	-1.1
	Oleuropein	-2.49
	Oreintin	-1.46
	Quercetin	-3.14
	Ursolic acid	**-6.63**
	Cynarin	-0.28
	Rutin	-1.43
1AGW	Gallic acid	-3.52
	Kaempferol-3-0-rutinoside	-0.69
	Mangiferin	-2.42
	Oleuropein	-1.06
	Orientin	-0.49
	Quercetin	-3.45
	Rutin	-0.06
	Steviol	**-6.81**
	Ursolic acid	**-6.15**
	Cynarin	0.45

4.4.7. Docking interaction between urosolic acid and 1r5k

The docking analysis between 1R5K and urosolic acid shows that one oxygen atom (atom red in color) of the ligand interacts with three hydrogen atom (atom green in color) of different aminoacid of the protein. Of the one oxygen atoms of the ligand interacted, one bond with hydrogen atom of the ASP 193 aminoacid with bond length of 1.93 Å. These distances are within the limits of molecular mechanics. A binding score of -5.11 and 3 hydrogen bonds proves the efficacy of urosolic acid in docking 1r5k.

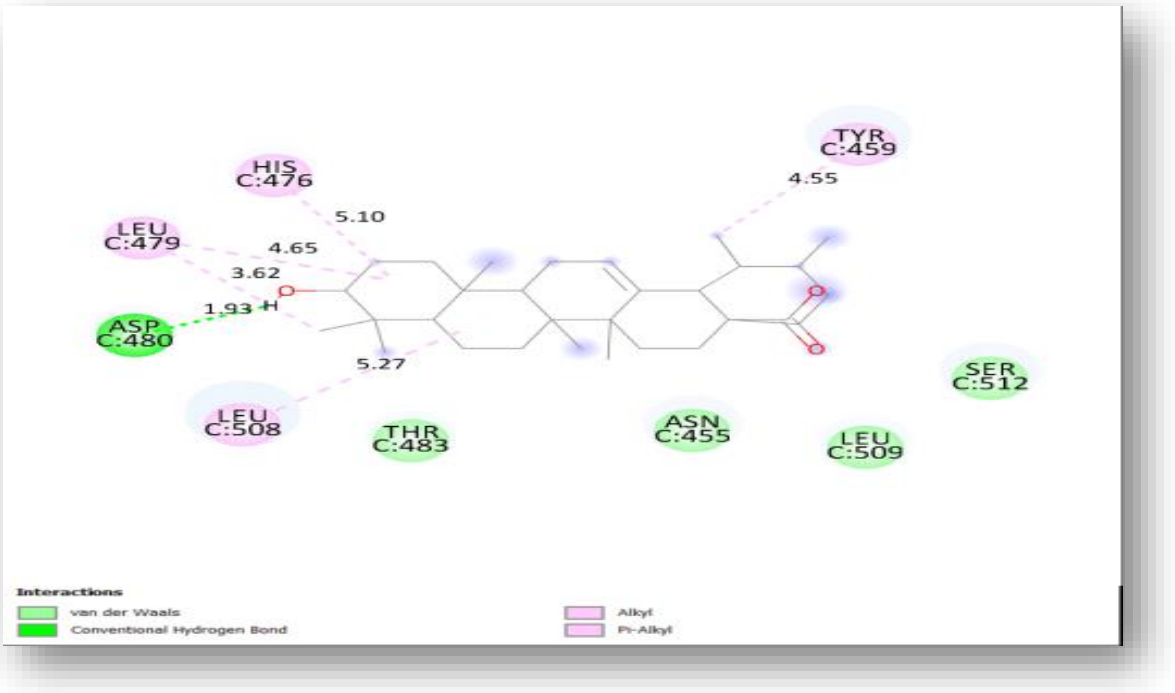


Fig.2 2D plot of ursolic acid and 1r5k

Table IX: 2D plot of ursolic acid and 1r5k

Protein complex	Amino acid	Bond length	Binding score	No.of hydrogen bond
1r5k and Urosolic acid	ASP	1.93	-5.11	1

4.4.7.Docking interaction between steviol and 1r5k

The docking analysis between 1R5K and Steviol shows that three oxygen atom (atom red in color) of the ligand interacts with three hydrogen atom (atom green in color) of different aminoacid of the protein. Of the three oxygen atoms of the ligand interacted, three bonds with hydrogen atom of the GLU 323, LYS 449 and ILE 386 aminoacid with bond length of 2.41,

2.42, and 2.78 Å. These distances are within the limits of molecular mechanics. A binding score of -6.56 and 3 hydrogen bonds proves the efficacy of steviol in docking 1r5k.

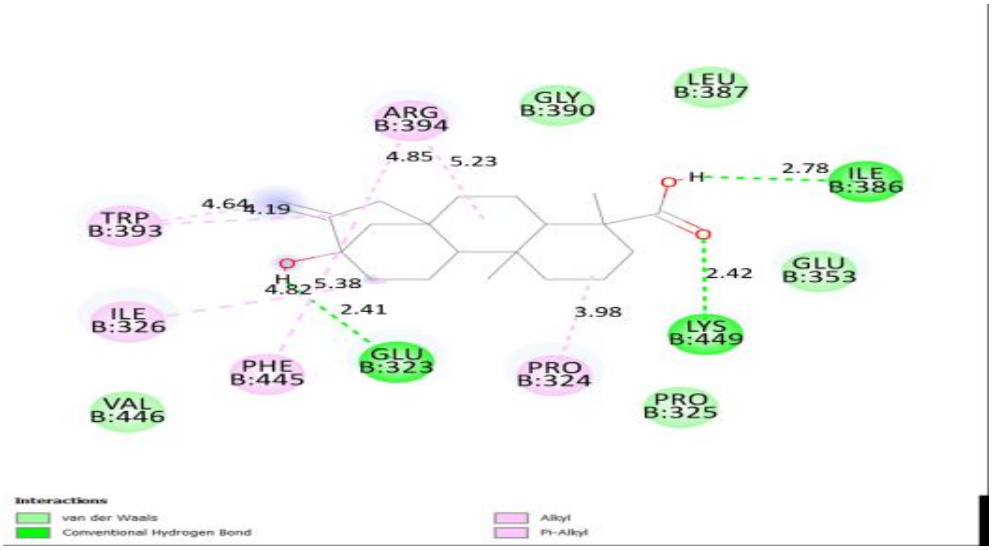


Fig. 3 2D plot of steviol and 1r5k

Table. X 2D plot of steviol and 1r5k

Protein complex	Amino acid	Bond length	Binding score	No.of hydrogen atom
1r5k and steviol	GLU	2.41	-6.56	
	LYS	2.42	-6.56	3
	ILE	2.78	-6.56	

4.4.7. Docking interaction between urosolic acid and 3poz

The docking analysis between 3poz and urosolic acid shows that three oxygen atom (atom red in color) of the ligand interacts with three hydrogen atom (atom green in color) of different aminoacid of the protein. Of the three oxygen atoms of the ligand interacted, three bonds with hydrogen atom of theVAL717, ASP 123 and aminoacid with bond length of 4.87, and 2.03 Å. These distances are within the limits of molecular mechanics. A binding score of -6.63 and 3 hydrogen bonds proves the efficacy of urosolic acid in docking 3poz

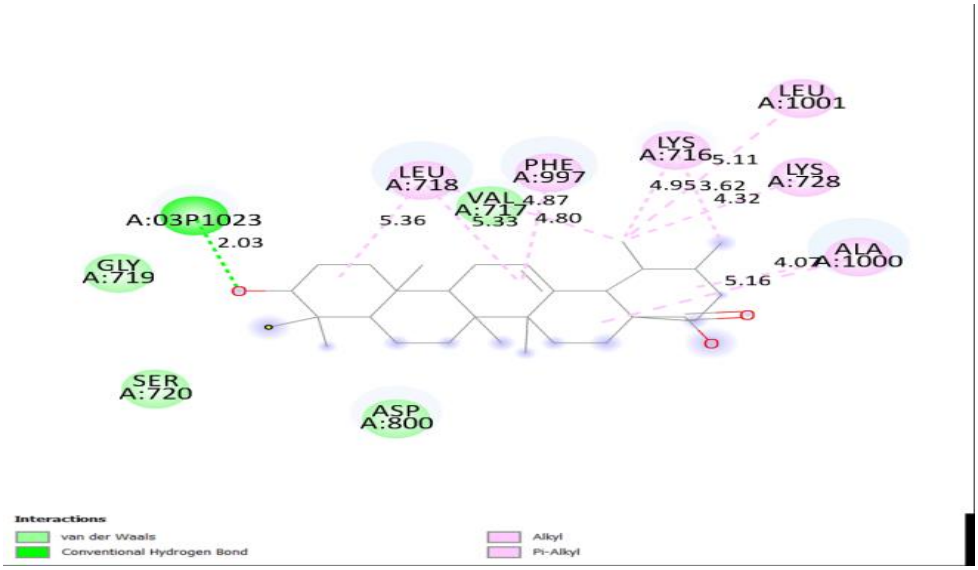


Fig. 4 2D Plot of Urosolic acid and 3poz

Table. XI 2D Plot of Urosolic acid and 3poz

Protein complex	Amino acid	Bond length	Binding score	No.of hydrogen atom
3poz and urosolic acid	VAL	4.87	-6.63	3
	ASP	2.03		

4.4.8. Docking interaction between urosolic acid and 3poz

The docking analysis between 3poz and urosolic acid shows that two oxygen atom (atom red in color) of the ligand interacts with three hydrogen atom (atom green in color) of different aminoacid of the protein. Of the two oxygen atoms of the ligand interacted, three bonds with hydrogen atom of the ALA 702, GLU 711, ILE 706 and aminoacid with bond length of 2.26, 2.18,2.00 and 2.05 Å. These distances are within the limits of molecular mechanics. A binding score of -5.34 and 5 hydrogen bonds proves the efficacy of urosolic acid in docking 3poz.

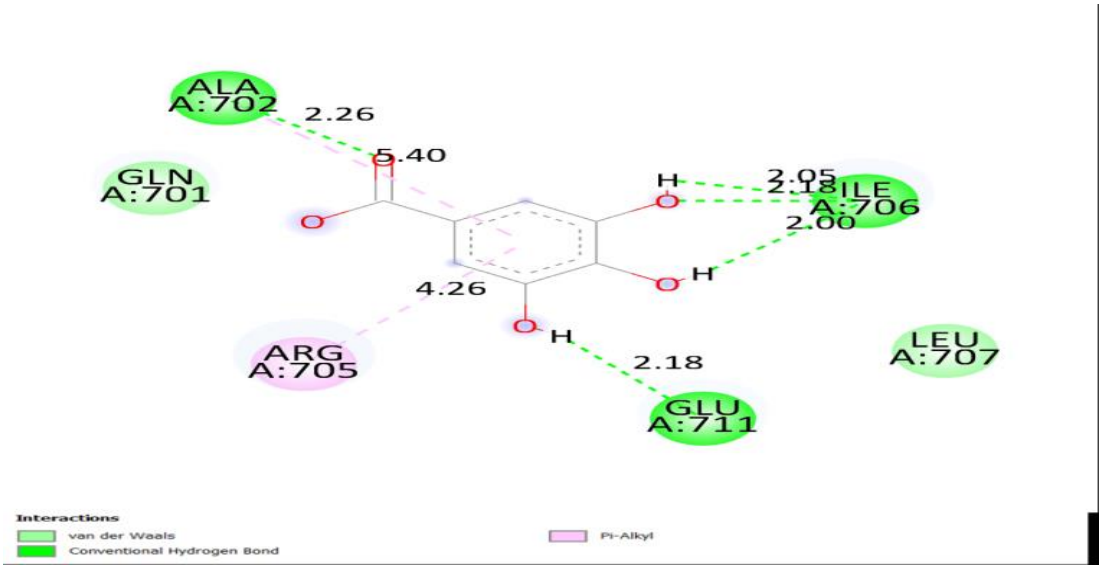


Fig. 5 2D Plot of Gallic acid and 3poz

Table. XII 2D Plot of Gallic acid and 3poz

Protein complex	Amino acid	Bond length	Binding score	No.of hydrogen atom
3poz Gallic acid	ALA702	2.26	-5.34	5
	GLU 711	2.18		
	ILE 706	2.00		
	ILE 706	2.05		
	ILE 706	2.18		

4.4. 13 Docking interaction between urosolic acid and 1agw

The docking analysis between 1agw and urosolic acid shows that two oxygen atom (atom red in color) of the ligand interacts with two hydrogen atom (atom green in color) of different aminoacid of the protein. Of the two oxygen atoms of the ligand interacted, three bonds with hydrogen atom of the TYR 654, LYS 656 and aminoacid with bond length of 2.83, 2.00 Å. These distances are within the limits of molecular mechanics. A binding score of -6.15 and 2 hydrogen bonds proves the efficacy of urosolic acid in docking 1agw.

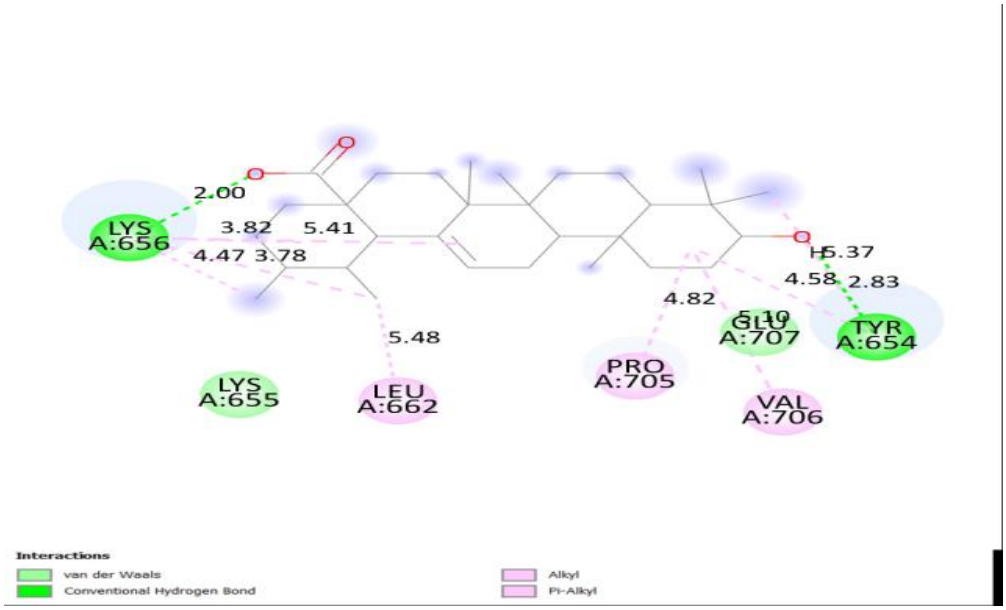


Fig.6 2D Plot of Urosolic acid and 1agw

Table XIII: 2D Plot of Urosolic acid and 1agw

Protein complex	Amino acid	Bond length	Binding score	No.of hydrogen bond
1agw Urosolic acid	TYR 654	2.83	-6.15	2
	LYS 656	2.00		

4.4.11 ADMET properties

After the molecular docking studies of 10 ligands with the fourtarget protein responsible for wound, the ADMET properties (absorption, distribution, metabolism, elimination and toxicity) of the compounds with better binding affinity were screened using the online tool pkCSMto predict their important pharmacokinetic properties. The results of ADMET properties of the 4 compounds with better binding affinity are summarized in Table IX.

Table XIV. Prediction of ADMET profile of the selected compounds

Parameter	Gallic acid	Quercitin	Steviol	ursolic acid
Absorption				
Water solubility	-2.56	-2.925	-2.907	-2.856
Caco2 permeability	-0.081	-0.229	1.347	0.539
Intestinal absorption (human)	43.374	77.207	98.759	60.492
Skin Permeability	-2.735	-2.735	-2.732	-2.735
P-glycoprotein substrate	No	Yes	No	Yes
P-glycoprotein I inhibitor	No	No	No	No
P-glycoprotein II inhibitor	No	No	No	Yes
Distribution				
VDss (human)	-1.855	1.559	-0.942	-1.36
Fraction unbound (human)	0.617	0.206	0.184	0.138
BBB permeability	-1.102	-1.098	-0.134	0.09
CNS permeability	-3.74	-3.065	-1.83	-2.472
Metabolism				

CYP2D6 substrate	No	No	No	No
CYP3A4 substrate	No	No	Yes	Yes
CYP1A2 inhibitior	No	Yes	No	No
CYP2C19 inhibitior	No	No	No	No
CYP2C9 inhibitior	No	No	No	No
CYP2D6 inhibitior	No	No	No	No
CYP3A4 inhibitior	No	No	No	No
Excretion				
Total Clearance	0.518	0.407	0.507	-0.043
Renal OCT2 substrate	No	No	No	No
Toxicity				
Ames toxicity	No	No	No	No
Max. tolerated dose (human)	0.7	0.499	-0.141	0.349
hERG I inhibitor	No	No	No	No
hERG II inhibitor	No	No	No	No
Oral Rat Acute Toxicity (LD50)	2.218	2.471	1.954	2.44
Oral Rat Chronic Toxicity (LOAEL)	3.06	2.612	1.948	0.491
Hepatotoxicity	No	No	Yes	No
Skin Sensitisation	No	No	No	No
T.Pyriformis toxicity	0.285	0.285	0.284	0.285
Minnow toxicity	3.188	3.721	0.64	-1.124

In the development of innovative drugs, compounds must have an advantageous ADMET profile. The pkCSM online server was employed to calculate *In silico* ADMET properties (Table 5). Caco-2 cells have been utilized generally as an *In vitro* model to study absorption efficiency through epithelial barriers related to cellular transport in the body due to their resemblance to small intestine epithelial cells. ADMET results are interpreted based on apparent standard value of all the properties which possess a certain limit for drug like. In the present study the high Caco-2 permeability was predicted to be > 0.90 where the intestinal absorption to be higher than 30%. This was considered that the drug candidates such as Quercetin, Steviol, and ursolic acid and Gallic acid got highly absorbed. Similarly, the HIA range from 32.274 to 94.539%, implying that they all tend to be easily absorbed in the gut. Most of the compounds displayed a value above 80%, indicating a good absorbance in the human intestine and the human VDss is low if it is below -0.177 L/kg and high above 1.559 L/kg. The compound steviol only exhibited

low human VDss. For a given compound, a logBB< -1 is poorly distributed to brain, whereas logBB> 0.3 indicates the high potential to cross the BBB and here the logBB indicates the potential to penetrate the CNS for the compounds like Quercetin, ursolic acid and Gallic acid.

Also, the Log Kp of all the screened phyto compounds were less than -2.7, revealing their high skin permeability. The P-glycoprotein extrudes toxins from the cells. From this analysis, Gallic acid and steviol were found to be substrates of P-glycoprotein. The other compounds were inhibitors of P-glycoprotein which could inhibit the P-glycoproteins when over expressed in the cell surfaces and prevent the excessive efflux of drugs.

The metabolism ensures the chemical biotransformation of a phyto compounds in the body, which plays a key role in transformation of drug compounds. In the body, drugs produce several metabolites which contribute to catalyze the reaction with various drug concentrations. It is important to consider their metabolism, which may show various physicochemical and pharmacological parameters. In this study, CYP2D6 and CYP3A4, the iso forms of CYP450, were utilized for metabolism evaluation. Cytochrome P450 is a detoxifying enzyme that plays a major role in drug metabolism because the major liver enzyme system is involved in phase 1 metabolism[19].Some selective CYP genes, including CYP1, CYP2, CYP3 and CYP4, were found to be involved in drug metabolism which causes the biotransformation in greater than 90% of drugs undergoing phase I metabolism. In the metabolism analysis, the compoundswere examined whether they were inhibitors or substrates of CYP2D6 and CYP3A4. Caffeic acid was the substrate of CYP3A4and CYP2D6. Clearance is a constant that indicates the relationship between drug concentration in body and the rate of elimination of drug. Therefore, all compounds showed a significant clearance value where the drug could be easily excreted from the body. Furthermore, it is necessary to examine whether the calculated compounds are non-toxic, because this plays a critical role in the selection of drugs. In the toxicity prediction, the toxic nature of all the ligands using various tests was analyzed. All the drug candidates in the present study were predicted to be non-toxic by the AMES toxicity test and Hepatotoxicity test. Additionally, the compounds were also non sensitive to skin. Maximum tolerated dose (Human) and Oral rat chronic toxicity (LOAEL) doses were given in the ADMET analysis. Overall toxicity analysis revealed that all the screened phyto compounds were non carcinogenic to rat, had less lethality, and werenon-hepatotoxic.The findings of present study suggest that the

compounds showed higher binding affinity with the wound proteins, non-toxic, good solubility and stability is proficient and competent for pursuing the wet experiments which hold its candidacy in therapeutics and drug discovery for wound.

5 DISCUSSION

Plants have been used throughout the world for its medicinal powers since ancient time (Chandran *et al*., 2020). India is a reservoir of numerous high-valued medicinal plants and is one of the major medicinal plants producing Asian country (Anand *et al*., 2019). The pharmacological properties of plants are based on their phytochemical components especially the secondary metabolites which are outstanding sources of value added bioactive compounds. The most important things for consumers about medications are purity, safety, potency, and efficacy.

The species of Morinda genus are one of the ethnic plants of tropical countries and are used in folk medicine since ages. Even though the use and prescription of the synthetic drug are increased remarkably, about 20–25% of active drug compounds were directly derived from the plants. *M. tinctoria* Roxb. is known for the medicinal and nutritional values. *M. tinctoria* Roxb. fruit juices have been used an alternative medicine for the treatment of arthritis, diabetes, muscle aches, menstrual complications, cancers, gastric ulcer, heart disease, and drug addiction. Apart from fruits, leaves and roots of *M. Tinctoria* Roxb. are used as astringent and deobstrent.

Liposomes are nanoparticulate systems that have been adopted by several researchers as the system of release of choice for the administration of drugs, vaccines and targeting of therapeutic agents. Because of their unique composition and structure, liposomes have a number of favorable features, including high biocompatibility, biodegradability, high drug transportability and easily adjustable handling properties (Zhu *et al*., 2014). Liposomes present as an option capable of transporting AgNPs (Barani *et al*., 2011). The conventional liposome composition consists of a lipid bilayer which may be composed of cationic, anionic or neutral phospholipids and cholesterol, which encloses an aqueous core. cholesterol is an important bio molecule for cell membrane function because of its ability to control the fluidity of the lipid membrane. Cholesterol is often added to phosphor lipid liposomes to improve stabilization (Aramaki*et al*., 2016). Both the lipid bilayer and the aqueous space may incorporate hydrophobic or hydrophilic compounds, respectively.

Thus, considering the importance of the constant search for new systems with potential wound healing and antimicrobial action, as an alternative to conventional treatments, this work proposes the development of liposomes loaded with *M.tinctoria* Roxb. leaves extract for wound healing.

Phytochemical evaluation is an efficient way to predict the plant drugs and discover various components present in different polar solvents. An extraction technique with superlative effectiveness with respect to time/yield ratio is fundamental to exactly quantify the phyto constituents (Farooq *et al*., 2018). Preliminary phytochemical screening is helpful in the prediction of nature of medicines and also useful for the discovery of various constituents existing in solvents of diverse polarity.

The qualitative phytochemical study indicated the presence of compounds such as alkaloids, carbohydrates, glycosides, saponin, phytosterol, phenols, tannins and proteins with maximum in the ethanol extract as compared to all other extracts. This was in agreement with the earlier studies. The present outcomes are almost similar to the results of Jain *et al*., 2014 who reported the presence of tannins, phlobatannins, terpenoids and glycosides in the ethanolic extracts of *M.tinctoria* Roxb..

Based on the results (i.e., the antimicrobial activity) the liposome loaded with *M. tinctoria* Roxb. leaves exhibited varying degree of inhibitory activity against the growth both gram positive and gram negative bacteria tested. The mean inhibition zone for the tested bacteria ranged from 5mm – 24mm indicating a remarkable antibacterial effect when compared with ampicillin the positive control, which ranged from 11mm – 17 mm. The infections caused by Proteus vulgaris and Streptococcus aureus, can be treated with liposome loaded with *M.tinctoria* Roxb. leaves as it exhibited the more inhibitory activity against the pathogen.

Generally, gram positive bacteria (*Staphylococcus aureus*) were more sensitive to liposome loaded with plant extracts because of the presence of a mesh like peptide glycan layer which is more accessible to permeation by the extracts (Tajkarimi *et al*., 2018). The resistance of the gram negative bacteria (*Klebseilla pneumonia, Escherichia coli*) could be attributed to its cell wall structure. Gram negative bacteria have a powerful permeability barrier, composed of a thin lipo polysaccharide exterior membrane, which could restrict the penetration of the extruding

liposome loaded with plant extract. It has been discussed earlier that gram negative bacteria are usually more resistant to the plant originated antimicrobials and even show no effect, compared to gram positive bacteria (Tajkarimi *et al*., 2018).

Natural antioxidants present in the plant extracts helped humans in the maintenance of health for thousands of years. “Antioxidant compounds like flavonoids, phenolic compounds and polyphenols scavenge free radicals. The antioxidants contain peroxide, hydro peroxide or lipid peroxyl and hence prevent the oxidative processes that lead to progressive ailments (Bouyahya *et al*., 2020). The mechanism by which antioxidants play their protective role can be classified into two types. First mechanism is referred to as hydrogen atom transfer, the free radical removes a hydrogen atom from the antioxidant, that itself becomes a radical. The second mechanism is based on electron transfer, the antioxidant can give an electron to the free radical and itself become a radical cation (Nayik, and Gull, 2020).

The present study showed a dose dependent scavenging power with maximum activity in the ethanolic extract of *M.tinctoria* Roxb. leaves (95.08%). The evaluation of DPPH scavenging activity is the most consistent assay for evaluating the antioxidant activity of the plant extracts. DPPH (di phenylpicryl hydrazine (C18H13N5O6, M=395.3) is a commercially available organic nitrogen radical. DPPH can only be dissolved in organic media; this condition becomes an important limitation when it comes to interpreting the role of hydrophilic antioxidants (Desmiaty *et al*., 2018). This study, the DPPH radical scavenging assay reveals the proton contribution to the lone pair electron of the radicals. DPPH displays a strong absorption maximum at 517 nm. The color change takes places to yellow from purple that indicate the formation of DPPH by the absorption of hydrogen from the antioxidant present in the sample. This reaction is stoichiometric in relation to the number of hydrogen atoms occupied. Hence, the antioxidant effect can be easily accessed by following the reduction of UV absorption at 517 nm (Abu-Darwish *et al*., 2018).The present study showed a dose dependent scavenging power with maximum in the ethanol extract. The potential of the scavenging ability has been mentioned in percentage. The efficacy increased with increase in concentration. In this study, the DPPH radical scavenging assay reveals the efficiency of the plant extracts in contributing a proton to the lone pair electron of the radicals.

Subsequent studies in the late 1990s that specified that poor pharmacokinetics and toxicity were significant causes of high late-stage failures in drug improvement. It has become broadly valued that these areas should be considered as first as possible in the drug discovery process (Rad *et al*., 2021). Lipinski's rule of five recognizes numerous critical properties that should be considered for compounds for oral delivery. In this study, for *In silico* analysis, total of 10 bioactive molecules obtained from literature analysis and were retrieved without any ambiguities from the Pubchem deposition (Harrad *et al*., 2018). Among the selected 10 compounds such as Kaempferol-3-O-rutinoside, Mangiferin, Oleuropein, Orientin, Quercetin, Rutin, Steviol, Ursolic acid, Gallic acid and Cynarin, only four compounds such as, Ursolic acid, Gallic acid, Quercetin, Steviol obeyed the Lipinski rule of five.

In the present study, bioactivity score was noted for enzyme inhibitor, kinase inhibitor, GPCR ligand, ion channel modulator, nuclear receptor ligand, and protease inhibitor. The good bioactivity score is an indication of the excellent pharmacological activity *In vivo* (Nath *et al*., 2014). The bioactive scores for the compounds can be assumed as active (bioactivity score > 0), inactive (bioactivity score < -5.0) and moderately active (bioactivity score: -5.0-0.0). From the bioactivity analysis of the compounds present in *M. tinctoria* Roxb., the results indicate that the selected compounds were moderately active as the score for each compound ranges from -5 to 0. Cynarin, steviol and orientin have exhibited high positivity indicating their high bioactivity**.** Our observation corroborates with the findings of Sytar *et al*., 2018 who reported that bowelled acid and its derivatives exhibit good bioactivity score for drug targets including nuclear receptor ligand, protease inhibitor and enzyme inhibition and thus expected to have excellent pharmacological activity *In vivo*. In another study, the antioxidant compounds present in Aloevera were screened in search of a new lead compound and found that dihydro coumarin ethyl ester showed good drug likeness score and bioactivity score, on comparison with other compounds and chosen as the best (Ahvazi *et al*., 2018). In another study, (Valli and Geetha, 2015) made an *In silico* prediction of bioactivity of flavonoids present in Erythrina varigata and showed that methoxy phaseolludin exhibited the highest score towards GPCR (G-protein-coupled receptors) ligand, nuclear receptor ligand and enzyme inhibitor activities among the sixteen flavonoids analysed. Similarly molecular properties and bioactivity score of alkaloids present in Erythrina varigata leaves were calculated using Molinspiration software. As per the comparative scores of fifteen alkaloids studied, alkaloid I exhibited the highest score towards

GPCR ligand, exhibited ion channel modulator, kinase inhibitor and enzyme inhibitor activities (Valli and Jayalakshmi, 2015). Hence, the present study also concludes that the compounds such as catechin hydrate and epicatechin will have outstanding pharmacological activity as they had good bioactivity score for nuclear receptor ligand, protease inhibitor, GPCR ligand, ion channel receptor and enzyme inhibition. Also ellagic acid will also expected to have active pharmacological properties as they have good bioactivity score for nuclear receptor ligand

The bioactive compounds identified in the *M.tinctoria* Roxb. leaves have previously reported to possess many biological activities such as antioxidant, anticancer, anti-inflammatory, antimicrobial, wound healing, anti- inflammatory and anti-diabetic activity. It is the most extensively used oil in therapy, both internally and externally, being recommended for the treatment of acute and chronic gastritis and enteritis, in disorders of the respiratory tract, and for inflammation of the oral mucosa (Balakrishnan, 2015).

Molecular docking is a method used to predict the binding orientation of small molecular drug candidates to their protein targets in order to predict the affinity and activity of the small molecule (Maresch *et al*., 2018). Recently docking ligands to receptors utilizing rational drug design is on the increase owing to few problems in the conventional methods of drug designing. Hence docking plays an important role in the rational design of drugs. Given the biological and pharmaceutical significance of molecular docking, considerable efforts have been directed towards improving the methods used to predict docking. In the present investigation, ten bioactive components have been selected from extensive literature search and docked against the four different wound healing protein. The selected bioactive components were docked with each of the four wound healing 1R5K, 1X7B, 3POZ and IAGW.

The result showed that Steviol and quercetin exhibited a significant docking interaction with wound protein, Oestrogen receptor – Beta (1X7B) with the docking score of -7.23 and -7.06 kcal/mol, the maximum negative score shows good binding energy . Gallic acid and Urosolic acid exhibited a significant docking interaction with wound protein, Epidermal Growth Factor Receptor (3POZ) with docking scores of -5.34 and -6.63 kcal/mol. Steviol and urosolic acid exhibited a significant docking interaction with wound protein, Fibroblast growth factor receptor (1AGW) with docking scores of -6.81 and -6.15 kcal/mol. Steviol and urosolic acid exhibited a significant docking interaction with wound protein, Oestrogen receptor – alpha

(1R5K) with docking scores of -6.56 and -5.11 kcal/mol. The best scoring against the target proteins was mean to be with better agonistic effect leading to wound healing potency. Low docking energy indicates high binding ability. Based on this observation, it can be concluded that Steviol can be considered as good drug candidate for wound healing treatments.

The present study is in line with the study of Sreejaya and Santhy (2015) who tried to find out drug candidates for breast cancer from the methanolic extract of Acorus calamus. Analysis by GC-MS of the extract revealed 14 compounds. These compounds were taken for docking studies using highly influential breast cancer proteins. Based on the results, ERBb1 protein was found to be a good target for breast cancer. Their study identified [(2R)-2-[(1S)- 1-hexadecanoyloxy-2- hydroxyethyl]-4-hydroxy-5-oxo-2H-furan-3-yl] as a ligand that act as a good inhibitor for breast cancer. Docking of 26 withaferin and 14 withanolides from Withaniasomnifera in to the PknG of Mycobacterium tuberculosis revealed that with anolide E, F and D and withaferin-diacetate 2 phenoxy ethyl carbonate were identified as potential inhibitors of PknG (Santhi and Aishwarya, 2011).

Based on this observation, it can be concluded that Steviol can be considered as good drug candidate for wound healing treatments.

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SUMMARY AND CONCLUSION

Medicinal plants are only source and an important contribution for primary healthcare during ancient times. Knowledge about use of medicinal plants for treating various diseases was highly valued among ancient civilizations. Until the mid-nineteenth century, plants were the main therapeutic agents used by humans and still have an important role in medicinal preparations. About 80% of people in developing countries depend on traditional medicine for their primary health care needs, because of their low costs, effectiveness, frequently inadequate provision of modern medicine, cultural and religious preferences. 80% of people in India use non-allopathic (Ayurveda, Siddha, Unani and Homeopathy) herbal based medicines for their healthcare which are collected from wild and cultivated sources.

Current estimates indicates that nearly 6 million people suffer from chronic wounds worldwide. Unhealed wounds constantly produce inflammatory mediators that produce pain and swelling at the wound site. Wounds are a substrate for infection and prolong the recovery of injured patients. Chronic wounds may even lead to multiple organ failure of death of the patients. Wounds are the physical injuries that result in an opening or breaking of the skin and appropriate method for healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin.

- In the present study, preliminary screening and qualitative phytochemical analysis of different solvents (petroleum benzene, ethanol, chloroform and aquaeous) revealed that the presence of eight major phytoconstituents in the ethanol extract namely, alkaloids, carbohydrates, glycosides, saponin, phytosterol, phenols, tannins and proteins.
- The free radical scavenging action is known to be one of the important mechanisms for measuring antioxidant activity. The highest scavenging efficacy of *M.tinctoria* leaves recorded at the concentrations of 10µl and 50µl with the inhibition percentage of 95.08% and 85.25%. The DPPH reducing activity of the *M.tinctoria* leaves was measured based on color change which was shown due to the reduction reaction.

- The assessment of antibacterial activity revealed that the liposome loaded with *M.tinctoria* leave extract exhibited the zone of inhibition in Escherichia coli (13 mm), *Pseudomonas auregenosa* (15 mm), *Staphylococcus aureus* (13 mm), *Proteus vulgaris* (14 mm) *Enterococcus feacalis* (13mm) and *Klebseilla pneumonia* (14 mm). The liposome loaded with *M.tinctoria* leaves extract exhibited the maximum zone of inhibition in *Pseudomonas auregenosa* (15 mm).
- The combination of ADMET prediction and drug-likeness has shown promising results *in silico* since the phyto compounds of *M.tinctoria* have improved pharmacokinetic properties and complied with all conditions of drug-likeness rules.
- Ligands identified from the literature search were subsequently taken in for docking studies with highly influential wound protein such as Estrogen Receptor alpha (1R5K), Estrogen Receptor beta (1X7B), Epidermal growth factor kinase receptor (3POZ), Fibroblast growth factor (1AGW). Out of various complexes docked using Autodock 4.2.8, Estrogen Receptor beta (1X7B) -steviol complexes showed high binding energy of -7.23 kcal/mol.

In conclusion, the available evidence suggests that *Morinda tinctoria*, commonly known as Indian Mulberry, has wound healing properties. The plant's traditional use in indigenous medicine for treating various ailments, including wounds, has been supported by scientific studies that demonstrate its ability to promote wound closure, reduce inflammation, and prevent bacterial infections. While more research is needed to determine the optimal dosage and administration of *Morinda tinctoria* extracts for wound healing, its potential therapeutic benefits make it a promising candidate for further investigation.

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