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Computational Screening of *Citrullus colocynthis*–Derived Phytochemicals for Anti-Acne Activity

by

Tooba Arif

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CERTIFICATE OF APPROVAL

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Tooba Arif

(MBS243001)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Zoya Khalid	COMSATS, Islamabad
(b)	Internal Examiner	Dr. Sami Ullah Jan	CUST, Islamabad

Dr. Sahar Fazal
Thesis Supervisor
March, 2026

Dr. Syeda Marriam Bakhtiar
Head
Dept. of Bioinfo. & Biosciences
March, 2026

Dr. Sahar Fazal
Dean
Faculty of Health & Life Sciences
March, 2026

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(Tooba Arif)

Abstract

A significant number of adults as well as teens experience with acne vulgaris, a chronic inflammatory condition of the pilosebaceous unit that commonly results in permanent dermatological and psychological issues. The investigation of plant-based treatments has been encouraged by the limitations of traditional acne treatments, such as negative effects and the appearance of resistance. The well-known medicinal plant *Citrullus colocynthis*, frequently identified as bitter apple, has been shown to have antibacterial, anti-inflammatory, and antioxidant qualities. As such, it is a potentially natural source of bioactive chemicals for the treatment of acne. The current work used an in-silico drug development technique to analyse bioactive compounds produced from *C. colocynthis* as possible therapeutic agents against acne. The PubChem database provided a total 25 bioactive chemicals, and their selection had been supported by a thorough literature review based on documented anti-inflammatory, antibacterial, and anti-acne properties. The molecular target was chosen to be Keratin 16(KRT16), a protein linked to aberrant keratinization and acne etiology. KRT16's three-dimensional structure was obtained from the AlphaFold repository. Lipinski's rule of five was first used to evaluate drug-likeness, and then ADMET analysis utilizing the pkCSM platform was used to analyse pharmacokinetics and toxicity. PyRx was used for molecular docking to find chemicals that had a significant affinity for the target protein. Lipinski's criteria were used as a primary filter for the 25 identified compounds, and those that satisfied the conditions were then subjected to ADMET analysis. Campesterol was identified as the lead compound because molecular docking research revealed that it had the best interaction with KRT16, with a binding ability of -7.3 kcal/mol and suitable ADMET characteristics. Molecular dynamics (MD simulation was implemented to further evaluate both the stability and dynamic behaviour of the campesterol–KRT16 complex. The findings of the MD simulation show stable complex formation, favourable root mean square fluctuation (RMSF), appropriate root mean square deviation (RMSD), and continuous intermolecular interactions during the simulation. These results indicate Campesterol's potential as a promising bioactive chemical from *C. colocynthis* for the production new

anti-acne treatments by verifying its strong binding stability as well as helpful conformational behaviour with KRT16.

Keywords: Acne vulgaris, *Citrullus colocynthis*, Anti-acne drug discovery, Molecular Docking, ADMET profiling, In silico pharmacology

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Abbreviations

DHEAS	Dehydroepiandrosterone Sulfate
DHT	Dihydrotestosterone
IGF	insulin Growth factor
IL	interleukin
KRT16	keratin 16
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
ROS	Reactive Oxygen Species
TLT	Toll like receptors
TNF	Tumour necrosis factor

Chapter 1

Introduction

Acne vulgaris is a common skin problem of pilosebaceous units affecting people throughout the world [1], [2, 3]. Acne ranks number eighth among skin disorders because it affects 9.4% of total world population. More than 85% of teenagers are affected by acne and the problem can carry over into adulthood thus accounting for two thirds of dermatological visits related to acne [4]. non inflammatory lesions such as open/black or closed/white comedones and inflammatory lesions such as papules, pustules, nodules or cysts can appear on skin which often leads to development of scar and pigments on skin which requires a long and persistent treatments [5]. Mostly lesions are formed on face areas, neck, back and chest. Neonatal acne, occupational acne, infantile acne, acne mechanica, chloracne, acne conglobata, acne fulminans are different types of acne that affect populations commonly. Some types of acne is also caused by drugs such as acne caused by anabolic steroids, lithium, corticosteroids and phenytoin. Clinically and histologically these types are similar to acne vulgaris but clinical symptoms and its degree of severity distinguish acne vulgaris from other types [6]. There is no method to completely stop the acne formation but it can be treated nicely and acne is also linked with financial expenses. Acne can get even more worsen by factors such as Genetic conditions, environmental variables which include temperature, pollution, humidity, sun exposure, mineral oils/halogenated hydrocarbons and, nutrition deficiency ,

hormonal state, stress, smoking, comedogenic medicines such as androgens, halogens, corticosteroids, bacteria, and cosmetics [7, 8, 11]. Symptoms such as discomfort, emotional attacks, deformity, and possibly permanent scars are caused by Acne vulgaris. Also, patients may go through anxiety and depression due to embarrassment faced by them in public which leads to mental distortion [9, 10].

There are multiple pathogenic factors of Acne such as increased sebum production, high proliferation of bacterial colonies, hyperkeratinization of pilosebaceous units and inflammatory processes [12]. The next section will describe the various causative factors and pathogenesis.

Follicle damage releases keratin, lipids, and C. acne into the neighboring dermis which often sets off the inflammatory response [13]. To cause inflammatory response and wound development, the host immune system reacts by activating a variety of cytokines, including IL-1β, TNF-α, and IL-8 [14]. *C. acnes*'s involvement in biofilm formation increases its resistance to topical antibiotics and host defenses, which leads to recurring and frequent acne [15]. Additionally, oxidative or cellular stress has been linked to the abnormal physiology of acne, where peroxidation of lipids and sebaceous gland impairment are caused by an irregular balance between reactive oxygen species (ROS) and antioxidants [16]. The detail nature of acne and the needs for multi-targeted therapy techniques are pointed out by these multifactorial factors [17]. Stress, food, hormone imbalances, and environmental contaminants are some of the internal and external factors that can impact the outbreak and intensity of acne [18]. Androgens fluctuations cause the sebaceous glands to become more active, which leads to an excessive production of sebum, which blocks pores and promote the growth of bacteria [19]. High intake of sugar and dairy protein-rich foods have also been associated to more severe acne, because possibly they affect insulin-like growth factor-1 (IGF-1), which stimulates the sebum production and keratinocyte expansion [20]. Furthermore, emotional stress studies reveal to have worse effect on acne by immunological disruption and pathways driven by cortisol [21].

Keeping in mind disease process and therapeutic strategies much research has been done on acne. Recent advances in acne treatments include the use of combine

treatments which particularly address root pathogenic factors of acne. Recent literature explains some topical applications and novel treatment strategies for successful target therapy of acne. These include topical retinoids that reduce abnormal hyperkeratinization of infundibulum and new topical retinoids having anti-inflammatory characteristics[22, 23]. The focus in acne treatment is to manage the existing lesions by reducing sebum production, abnormal hyperkeratinization in pilosebaceous units and Propionibacterium infection. Therefore, the acne treatment options mostly includes using antibacterial and anti-inflammatory drugs [24–26]. Drugs can be administered through any of the following routes i.e. systemic, oral or topical. Some non-drug treatments can also be applied such as cryotherapy, comedone extraction, optical therapy, and intralesional corticosteroids. Combine application of topical and oral strategies can prove more effective in treatment of acne pathogenesis [24].Topical treatment is typically applied on the disease area thereby reducing systemic absorption and increasing the exposure of pilosebaceous units. Topical treatment formulations involve creams, gels, washes, lotions and solutions which can treat mild to moderate acne[27, 28]. Among these retinoid is the best option used in treatment of acne. Skin irritation is a common symptom seen in many patients who are treated with topical administered medications. Treatment may continue for 6–8 weeks or last for several months to years [29]. Furthermore, there are oppositions for hormonal treatment such as hormone pills and antiandrogens among certain group of people, and these treatments pose a risk of mood swings, blood clot disorders, and hormone irregularity [30]. Strong retinoid isotretinoin is exclusively used for severe and unreactive acne; however, it has serious bad effects, such as liver toxicity, mood swings, and birth defects risks [31]. Apart from the problems linked with drugs, many conventional therapies focus on limited elements of acne development, which often leads to temporary relief [32]. This highlights how significantly more safer, multi-targetic resisted substitutes are required, like bioactives derived from plants that have milder impacts and function as oxidative stress reducer, antimicrobials, and anti-inflammatory factors [33]. Another important issue in the treatment of acne is high chance of recurrence after the treatment is withdrawn along with some side effects and antibiotic resistance [34]. Patients after quitting antibiotics usually relapse within weeks or

months thus lacking longterm efficacy. Multiple treatments are required due to cyclic nature of acne leading to cumulative toxicity and discontent of patient [35]. Also, *C. acne* strains continuously develop resistance mechanisms therefore misuse of antibiotics in acne treatment can also leads to multidrug resistance [24].

Application of synthetic treatment is restricted for people with sensitive or allergy prone skin. Using numerous topical medications leads to contact dermatitis, burning and stinging, thereby lowering pateints adherence and life quality [25]. Male patients and those suffering from hypertension, breast cancer risk or PCOS patients are not allowed to get hormonal therapy [26]. Use of natural plant-based alternatives is becoming more famous because they are less irritating, thus they prove efficient in terms of safety, tolerance and developing resistance. The use of medicinal plants has been widely considered since ancient times for treating different skin disorders such as acne, psoriasis, eczema and wounds [28]. Due to presence of secondary metabolites such as alkaloids, terpenoids , flavonoids and phenolic compounds, these plants have wide spectrum of biological activities[29]. Medicinal plants are different from synthetic drugs in the way that the phytochemical they contain usually act on multiple cellular sites instead of targeting a single pathway, which make them significant in managing multifactorial states like acne [36]. Due to increasing frequency of drug resistance and worse effects linked with conventional therapies, the interest in plant dermatological treatments is increasing day by day [37]. Natural products are mostly well tolerated by skin because they have less toxicity and also exhibit some extra advantages such as antioxidant and wound healing properties making them useful in treatment of acne [38]. Also, the extract of various plants has been validated scientifically because of their ability to prevent *C. acne*s , reduce pro-inflammatory cytokines and control sebum production [39]. Recent research studies have determined special medicinal plants with anti-acne ability including tea tree (*Melaleuca alternifolia*), neem (*Azadirachta indica*), green tea (*Camellia sinensis*) , and turmeric (*Curcuma longa*) [40]. These plants containing phytochemical compounds act as basis for developing safe, novel and more powerful acne treatments. Consequently, less studied botanicals such as *C. colocynthis* are attracting the interest of researchers due to their unique anti-microbial and anti-inflammatory abilities [41].

Medicinal plants are considered beneficial due to their eco-friendly and cost-effective nature along with pharmacological qualities, which makes them significant in resource-limited healthcare environment [42]. Evidence based studies supports the involvement of phytotherapy in modern dermatological treatments, thus confirming that plant-based chemicals can act collaboratively with standard drugs or acts as template for further artificial derivatives [43]. This teamwork not only increase therapeutic performance but also help in reducing essential dosage and lesson worse effects related to drug [44]. Essentially, various plant components including polyphenols, terpenoids, amd alkaloids have biofilm breaking abilities which are explored efficiently to resist microbial resistance in skin infections [45]. Phytochemicals that have ability to inhibit biofilm production of acne, decline oxidative stress, and downregulate inflammatory mediators such as IL-1 β and TNF-α are of keen interest in acne management [46]. these compounds also have some additional benefits like skin regeneration, collagen strengthening, and promoting wound healing process [47]. Bitter apple, scientifically known as *Citrullus colocynthis*, is a member of the Cucurbitaceae family and has long been used medicinally to treat a variety of conditions, including skin conditions, diabetes, inflammation, and infections [48]. Different parts of the plant such as its fruit pulp, seeds, roots, and leaves are rich in variety of bioactive compounds.

Among them, cucurbitacins (particularly B, D, and E) are well investigated triterpenoids renowned for their following properties like anti-inflammatory, antibacterial, and antioxidant effects [49]. These substances have been documented to decrease the expression of pro-inflammatory cytokines like TNF-α and IL-6 and block microbial enzymes and neutralize oxidative stress mechanisms which directly connect to acne etiology [50]. In addition, the saponins found in *C. colocynthis* damage microbial cell membranes and biofilms leading to boosting of antibacterial activity against resistant strains like *C. acnes* [51]. Many studies show that extracts of *C. colocynthis* have sebostatic and wound-healing properties, which may be useful in reducing sebum production and accelerating the process of healing after acne [52].

Its antioxidant flavonoids help to scavenge reactive oxygen species (ROS), which

are typically found in inflammatory acne lesions [53]. Additionally, antibacterial effectiveness of *C. colocynthis* against number of bacteria and fungi, such as Staphylococcus aureus, Pseudomonas aeruginosa, and dermatophytes, has been verified in both in vitro and in vivo models [54].

Because of these characteristics, *C. colocynthis* is a promising option for more research as a natural, multi-targeted anti-acne drug, particularly through docking-based and computational studies.

At the end, the restrictions of current Acne vulagaris therapeutic strategies emphasize the exploration of new, cost effective and targeted treatments plans and as *C. colocynthis* has its rich history in ancient medicine and this plant also possess strong anti-inflammatory and anti-microbial properties so it presents a most promising but underexplored reservoir of bioactive chemicals that could be used in acne treatments.

This study aims to employ the In silico approach using molecular docking to screen the phytochemical constituents of *C. colocynthis* against the molecular targets involve in pathogenesis of acne. Together computational biology and ethnopharmacological insight this study aims to identify specific lead compounds which will provide key treatment for acne vulgaris, thus establishing a foundation for target development of novel therapeutic agents against acne vulgaris using a natural source.

1.1 Problem Statement

Acne vulgaris is a common dermatological condition with limited effective treatments and potential side effects. Conventional acne therapies are often limited by resistance, tolerability, and adherence issues, highlighting a need for novel treatments. While *C. colocynthis* is traditionally used for properties relevant to acne pathogenesis, its specific bioactive components are poorly defined. There is a need to employ rational drug discovery methods to validate its use, pinpoint specific bioactive compounds, and elucidate their mechanisms of action.

1.2 Aim

To identify potential lead compounds from *C. colocynthis* for acne vulgaris through the systematic in silico docking of its phytoconstituents.

1.3 Objectives of Study

i. To identify and prepare the key bioactive compounds from *C. colocynthis* from phytochemical databases.

ii. To retrieve and prepare the 3D structure of Human KRT16 protein.

iii. To perform molecular docking and determine molecular interactions that governs binding of protein and ligands.

iv. To identify and prioritizing lead compounds based on ADMET.

Chapter 2

Literature Review

2.1 Acne Vulgaris

Acne vulgaris is a long-term inflammatory condition that affects the pilosebaceous unit, which includes hair follicles and its linked oil glands. It is one of the most common skin diseases that occurs worldwide and affect people of all ages, though it is especially common among teenagers. In general adolescents' acne develops through a combination of different factors making it a complex condition. Its progression involves excessive keratinization of the skin, over production of sebum by the sebaceous glands, colonization of skin by microbes such as *Cutibacterium acnes* and activation of inflammatory responses. Even though these mechanisms are well developed the exact triggers that infinite the disorder is still not fully understood [55].

2.1.1 Prevalence of Acne

With an approximate global frequency of 9.38% throughout different age segments, acne vulgaris is the eighth common dermatological condition globally, according to the Global Burden of Disease Study. Estimates of the number of cases of acne in teenagers range from 35% to almost 100%, depending on the country and age group

[56]. Increased presentation to healthcare institutions and greater knowledge may be responsible for the increasing frequency of acne in adults. Acne may be more common in people who consume high-glycemic diets [57].

2.1.2 Causative Factors and Pathogenesis of Acne Vulgaris

The conventional root cause of acne vulgaris is thought to be largely influenced by a few causative variables. Elevated sebum secretion amounts, endocrinological variables such androgens, abnormal keratin production in the follicular infundibulum, microbial spread, and subsequent inflammation are the causes of chronic acne skin disorders, as previously suggested [58].

2.1.3 Raised Sebum Synthesis

One of the main factors contributing to the development of acne is the rise in sebum creation within the hair follicles. recent research shows that androgen hormones, particularly testosterone and insulin growth hormone (IGH-1), boost the production and release of sebum [59]. Excessive sebum production is a crucial factor that should be taken into consideration in individuals with acne vulgaris since there is a definite association between it as well as the severity and incidence of acne lesions [60–62]. Atypical pilosebaceous follicle hyperkeratinization: Single-cell keratinocytes frequently shed into the lumen by healthy follicles, where they are eventually removed. meanwhile, keratinocytes over proliferate and are not shed into the lumen in acne sufferers, which causes irregular desquamated corneocytes to accumulate in the pilosebaceous follicles together with lipids and monofilaments [63–65].

2.1.4 Overgrowth of *Propionibacterium acnes*

Another agent that causes acne is Propionibacterium, which contributes significantly to the pathogenesis of inflammation-induced acne. Since sebaceous follicles

generate plenty of sebum and offer a great anaerobic environment for bacterial development, *C. acnes* is an anaerobic, lipophilic, gram-positive bacterium that thrives to colonize there [66]. A lipase enzyme secreted by *P. acnes* breaks down sebum triglycerides to fatty acids and glycerol, which can cause skin irritation and comedone [67].

2.1.5 Inflammation Acne

The process of inflammation starts when the body's immune response recognizes *P. acnes*. Strong inflammatory effects from *P. acnes* could result in the production of chemostatic agents such as neutrophils, macrophages, and lymphocytes. These circumstances also lead to follicular rupturing, injury, and the release of lipids, fatty acids, and bacteria into the dermis layer. Lesions of inflammation such as ulcers which includes pustules, nodules, cysts, and papules will result from these mechanistic activities. Compared to inflammatory lesions, non-inflammatory lesions are smaller and contain less pus [68, 69]. Reactive oxygen species (ROS), which harm the follicular epithelium and exacerbate acne inflammation, have also been found to be produced by neutrophils. As a result, the follicular materials are released into the superficial skin layer, causing a range of inflammatory breakouts [70].

2.1.6 DNA Methylation

Under stressful conditions in environment, epigenetic modification which acts as the meeting point of heredity and the environment, can change how genes are expressed. Because of its role in the processes of inflammatory, autoimmune, and malignant skin disorders, DNA methylation is one of the well-researched types of epigenetic modification and is receiving more interest in the field of dermatologist. It has been demonstrated that DNA methylation contributes to the development and course of inflammatory skin conditions as psoriasis, atopic dermatitis, hidradenitis suppurativa, and others. Acne vulgaris is largely influenced

by epigenetics, which may provide information on its biological causes and possible treatments [71].

2.1.7 Types of Acne Lesions

Acne conglobates, acne fulminans, acne rosacea,acne cosmetica, acne excoriee, acne medicamentosa, acne chloracne, and acne mechanica are among the several types of acne [72, 73]. However, 99% of all occurrences of acne are acne vulgaris, which is the most common kind. It is distinguished by two distinct kinds of lesions: inflammatory papules, pustules, nodules, and cysts, and by non-inflammatory, open, and closed comedone. There are two forms of comedones: closed comedones are called whiteheads, and open comedones are called blackheads [74].

2.1.7.1 Blackheads

Overproduction of oil and cellular debris clogging the roots of hair cause blackheads, which are non-inflammatory acne lesions. Because the skin's surface continues to be apparent and has a dark appearance, like black or brown, a blackhead is known as an open comedo. Mild acne known as "blackheads" typically affects the face, arms, chest, neck, back, and shoulders [75].

2.1.7.2 Whiteheads

When oil, germs, and skin cells obstruct the entrance of hair follicle pore spaces, the skin forms whiteheads, which are tiny lumps and non-inflammatory acne lesions. Because the lumps are white and closed, whiteheads are also known as closed comedones. Although they can appear anywhere on the body, whiteheads generally most common in the T-zone, which comprises the forehead, chin, and nose [75].

i. Papules

Inflammation, which appears as tenderness, heat, itching, and discomfort, redness, is the reaction of healthy skin tissue to germs, excess oil production, and excess androgen activity. These inflammatory lesions, called papules, are thought to constitute a transitional stage between inflammatory and non-inflammatory lesions. Papules appear on the skin as a small, pink lump that is usually not packed with pus and has a diameter of under five millimetres [75].

ii. Pustules

When dead skin cells and extra oil block the pores, little lumps and inflammatory lesions are formed. They are filled with pus or fluid in the middle. They frequently appear as red, inflamed skin encircled by white pimples. Although they can appear anywhere on the body, pustules are most common on the shoulders, chest, back, face, neck, underarms, pubic area, and hairline [75].

iii. Nodules

When bacteria, extra oil, and cellular debris block the pores, acne nodules-a serious kind of aggressive acne-develop. This kind of mixture typically results in whitehead or blackhead comedone, but if the infection gets below the surface layer of the skin and damages the pores, the region around it may swell and turn red, giving the appearance of a little bump. Acne nodules can persist for weeks or months and cannot be treated with over-the-counter drugs alone. Similar to papule acne, nodular acne typically appears on the chin or jawline of the face and has a diameter more than 5 to 10 mm [75].

iv. Cysts

A serious type of inflammatory acne, cystic acne develops under the skin when clogged pores brought on by the development of bacterial cells, dry skin cells, and oil (see Fig. 2). The most afflicted individuals are those of all ages with oily skin. Large, painful, white or red lesions filled with pus are the usual appearance of cysts,

which can occasionally leave scars. Although it may develop everywhere on the body, the face, arms, shoulders, back, chest, and neck are the areas most commonly affected by cystic acne. Both inflammatory and non-inflammatory manifestations of acne are common in patients with cystic acne [75].

2.1.8 Pathophysiology of Acne

The pilosebaceous unit, which is made up of hair, a follicular channel lined with epithelium, and a variety of sebaceous organs, is the region where the multifactorial pathogenesis of acne begins [76]. The pathophysiology of skin breakout depends on number of significant factors, including follicular hyperkeratinization followed by *Propionibacterium acnes* proliferation within the follicle, sebaceous organ expansion associated with seborrhea, alteration in the structure of sebum lipids, intense phases other than safe reaction, dysregulation of the chemical microenvironment, and cooperation with neuropeptides [77, 78].

It has previously been proven that androgen levels and sebum production in acne vulgaris are related. The first reason behind acne is the androgen-induced enlargement of sebaceous glands resulting in greater sebum production [13]. The sebaceous glands contain steroid-metabolizing enzymes that transform DHEAS into DHT. In addition, testosterone is converted to the more dynamic DHT by two kinds of 5-α-reductase isozymes, type 1 and type 2, which are expressed in the scalp, chest, sebaceous organs, genital and urinary tissue, dermal papillae, and hair follicles [79]. Excessive manufacturing of sebum results in blockage of the pilosebaceous unit and increased turnover of cells in the follicular canal. Furthermore, pilosebaceous follicles are surrounded by macrophages and fiery middle individuals that communicate Toll-like receptors (TLR2) on their surface in the second part of pathogenesis. When TLR2 is activated, many cytokines are generated, including granulocyte macrophage-colony promoting factor (GM-CSF), interleukin-1 (IL-1), and IL-8 [80]. Keratinocyte hyperproliferation is subsequently caused by this mechanism, which initiates and propagates the inflammatory response. The pilosebaceous unit's retention of desquamated keratinocytes initiates follicular halting

and obstruction, which causes the follicle's normal design to be decimated and a delicate walled cystic injury-the pimple-to grow.

The microcomedo wall eventually breaches because keratinocytes and sebum continue to accumulate, causing discomfort [81]. Oily fittings that include a mixture of keratin, sebum, bacteria, and the superficial layer of melanin that may appear as a zit or white head are what cause pimples. "Black heads" or uncovered pimples are blisters that erupt through the surface of the skin and have a core black look because tyrosinase oxidizes tyrosine to melanin. The enlargement of the follicle with poorly desquamated keratinocytes and sebum is what causes "white heads" or closed wounds, which persist under the skin's surface and appear as closed follicles [82, 83].

These wounds can treat a papule, pustule, knob, and growth, depending on the severity of pathologic conditions. *Propionibacterium acnes* is an anaerobic Gram-positive bacterium that generates propionic and acetic acid. Since pimples provide a lipid substrate for anaerobes to feed on, many *P. acnes* are discovered in the follicular interior of pimples. *P. acnes* range between 0.4 to 0.7 μm wide and 3 to 5 μm long, with a relatively thick peptidoglycan cell wall and cytoplasm rich in ribosomes, according to ultrastructural observation [13].

By starting supplementation and converting sebum fatty oils into unsaturated fats that irritate the follicular wall and surrounding dermis, *P. acnes* contribute to the development of incendiary acne. It also chemotactically attracts neutrophils and distributes exoenzymes [84].

In addition to producing proteases, lipases, and hydrolases that exacerbate irritation and tissue destruction, *P. acnes* also create stress proteins that burst pimples and act on TLR-2 to trigger a reaction of inflammation. Cytokines like IL-6 and IL-8, which are believed to produce hyperkeratinization, cell adhesion, follicular blockage, and inflammation, may be expressed by follicular keratinocytes and macrophages as a result. Acneiform sores such as papules, pustules, and knobs are caused by follicular disruption and vascular and cell events of fiery response [85].

2.2 Traditional Medications for Acne Treatment

Regulating sebum production, aberrant hyperkeratinization of the pilosebaceous follicles, and propionibacterium infection are the main goals of the therapy options for acne vulgaris. Therefore, anti-inflammatory and antibacterial medications [86–89] are the primary treatment options for acne. These medications can be given topically, systemically, or orally, or physically using non-drug treatments like optical therapy, cryotherapy, comedone extraction, cyroslush therapy, and intralesional corticosteroids. However, when it comes to the pathophysiology of acne, combination of both treatments ; typical and oral works better [86, 87].

The benefit of topical medicines is that they are applied topically to the problematic area, enhancing interaction with the pilosebaceous follicular unit and reducing systemic absorption. Creams, gels, lotions, solutions, and washes are just a few of the various topical preparation formulas. Topical medications are commonly used to treat mild to severe acne [90, 91]. In addition to retinoids, individuals with acne are treated topically with antibiotics. Topically applied anti-acne drugs frequently cause irritation in the skin as a side effect. Topical therapy might persist for several years or only six to eight weeks [92].

2.2.1 Retinoids

The most popular first treatment for both inflammatory and non-inflammatory acne is topical retinoid therapy [86, 93]. Reducing sebum production, controlling the development of comedones, repairing the damaged epithelial layer, treating hyperpigmentation and scarring, preventing the formation of acne lesions, and controlling the growth of existing comedones are the main goals of retinoids. However, treating acne with this treatment is a drawn-out process that takes longer than three months. Dryness and skin sensitivity are two drawbacks of topical retinoids. Retinoids including tretinoin, adapalene, and tazarotene are frequently used to treat acne [75].

2.2.1.1 Tretinoin

This medication has anti-inflammatory qualities and is derived from vitamin A. This drug is typically used in combination with other retinoids to treat acne vulgaris by normalizing the epithelial layer, which prevents clogging of pilosebaceous unit and lowers sebum production. For more than thirty years, it has been used topically to treat acne. For the treatment of acne, it is sold as ointments, gels, and creams [94]. When used orally to manage leukemia, the adverse effects include headaches, skin that is dry, thinning hair, itching, and muscular aches, but when used topically, they are modest and produce sun sensitivity and redness [95].

2.2.1.2 Adapalene

This topical retinoid medication is mainly employed to treat people with mild moderate to serious acne. It is regarded as the initial course of action for acne since it has greater benefits than other retinoids like Tretinoin and Tazarotene [96]. It lessens the irritation brought on by acne and the hyperkeratinization of pilosebaceous follicles. There are very few adverse effects, such skin redness, inflammation, and irritation [97].

2.3 Combinational Topical Treatments

Blended topical therapy offers several advantages. Benzoyl peroxide, salicylic acid, niacinamide, azelaic acid, and dapsone are other topical medications used in combinations of therapy for acne.

2.3.1 Benzoyl Peroxide

Benzoyl peroxide can be utilized to treat mild to severe acne because it has antibacterial capabilities and functions as a topical non-antibiotic disinfection [98]. BPO has a bactericidal effect on *P. acnes* because it generates free oxygen, which

aids in the breakdown of bacterial proteins [99, 100]. BPO monotherapy can be used to treat acne patients for 6–8 weeks, however in order to limit *P. acne* species resistance and increase therapeutic efficacy, BPO is used in conjunction with topical antibiotics [101]. The most frequent side effects of BPO are irritating the skin, peeling, itchiness and redness [102, 103].

2.3.2 Salicylic Acid

Salicylic acid is a beta hydroxyl molecule which is composed of bacteriostatic, fungistatic, and anti-inflammatory qualities. When used topically, it keeps pores clean and helps to exfoliates the skin to lessen acne. Salicylic acid is safe to use, although it might occasionally irritate, itch, and dry up the skin [104]. It is mostly used for treating minor acne. Salicylic acid is a common ingredient in several over-the-counter medications used to treat acne [105]. Patients with acne who received supramolecular salicylic acid therapy had considerable improvements, and their microbial population was altered to resemble that of people without face acne [106].

2.3.3 Niacinamide

Nicotinamide, which is made up of niacin and nicotinic acid, is a kind of vitamin B3. It helps people with acne by reducing oil and sebum production and preserving the skin from getting acne [107]. Due to its anti-inflammatory qualities, niacinamide can be employed to treat mild to severe acne. It assists acne sufferers in recovering from wrinkles, redness, fine lines, and damage caused by the sun. Niacinamide is utilized in skincare products and acne remedies because of its healing and helpful qualities [108, 109].

2.3.4 Azelaic Acid

Azelaic acid is a naturally occurring phytochemical derived from barley and wheat and shows strong antibacterial, anti-inflammatory, anti-keratolytic and anti- oxidative properties [110]. The most prevalent negative effects of azelaic acid include

itching, breathing difficulties, skin redness, and burning. Acne and other skin conditions including skin whitening and hyperpigmentation can be treated with azelaic acid [111].

2.3.5 Dapsone

Although dapsone (diaminodiphenyl sulfone) possesses antibacterial and anti - inflammatory functions, it is still unclear how exactly it works to treat acne. It has recently been shown that mild to moderate acne may be treated by dapsone's antibacterial, immunomodulatory, and anti-inflammatory qualities [112]. Dapsone gel (5%) helps lessen the two inflammatory and non-inflammatory acne blemishes. This drug is more suited to use in underdeveloped countries due to its lower cost. However, it is not recommended as the primary indicator therapy for acne vulgaris [113, 114].

2.4 Systemic Treatment

Topical retenoid is regarded as primary treatment of acne because of microcomedo significant role in expansion of inflammatory as well as non-inflammatory lesions. When individuals' acne does not respond to topical therapy, manifests as nodules on the skin, or leaves scars, oral systemic treatment is recommended. For acne sufferers to avoid psychological and social shame, systemic therapy is crucial. The most popular systemic therapies for acne symptoms include prescription oral antibiotics, hormonal medications, and isotretinoin [115].

2.4.1 Retinoids

Isotretinoin is most commonly used drug in primary treatment of acne derived from vitamin A. Primarily this drug was the only medicine which have ability to reduce acne when used for long time patients who complain of not responding to oral or topical medications may also find it helpful. Therefore it is regarded as

first line treatment for most severe acne found on face, neck and trunk that also produce scars on skin [116].

2.4.2 Antibiotics

When acne is moderate to severe, inflammatory, resistant to topical therapies, or covers significant parts of the body, antibiotics given by mouth are typically recommended [117, 118].

Acne is frequently treated with intravenous antibiotics such as erythromycin, clindamycin, azithromycin, roxithromycin, fluoroquinolones (levofloxacin), tetracyclines (doxycycline, minocycline, and lymecycline), and co-trimoxazole [86, 103].

These antimicrobial medications inhibit *P. acnes* growth and the production inflammatory mediators. The effectiveness of the therapy is determined by the antibiotic's ability to enter the lipid environment of the pilosebaceous follicles within the dermis, where *P. acnes* colonizes [119].

Due to their cost-effectiveness, anti-inflammatory, and antibacterial qualities, tetracyclines are frequently used in the management of acne. Because of their anti-inflammatory qualities, less gastrointestinal distress, and increased lipid solubility, which enables them to more successfully enter the pilosebaceous follicle, doxycycline and minocycline are preferred over tetracyclines [120].

The success rate of azithromycin in curing acne has not been thoroughly investigated by the researchers. Erythromycin and clindamycin have modest anti-inflammatory effects, which lowers *P. acnes* levels [121].

When treating acne vulgaris, giving these antibiotics on a regular basis for an extended period of time increases resistance and reduces their utility. Combined therapy is now preferred in order to reduce resistance and increase effectiveness. When treating acne, oral antibiotics are combined with topical medications such as retinoids or benzoyl peroxide, and the course of treatment is restricted to 12 weeks [86].

2.4.3 Hormonal Treatment of Acne

Hormonal therapy was selected as an innovative approach to treating acne in adult females and adolescents. Although sebaceous glands are androgen-dependent, the action of testosterone on them can be treated with hormone treatment [122]. These hormones are frequently used as oral contraceptive pills. The creation of sebum, which is initially triggered by testosterone, is inhibited by these contraceptive hormone tablets. It reduces the quantity of physiologically active free testosterone in the bodies of women by increasing the development of sexual hormone-binding globulin [123].

Oral birth control pills alone or in combination with other therapies can be used to treat acne in women. The most advised course of therapy for acne in females with hormonal anti-androgens is at least 12 months, if not more, as the positive benefits of these medications only become noticeable after a 3–6 month treatment period [124]. An androgen receptor blocker called spirolactone is used in combination with oral contraceptives to lessen inflammation brought on by female acne [86].

2.5 Current Developments and Commercial Goods

New avenues for research and development, such as blocking the mechanistic route or mechanism associated with acne creation, have been become possible by recent scientific improvements in our understanding of the intricacy of acne. Recent research has focused on receptors, cytokines, chemokines, and other proinflammatory mediators to regulate these pathways. Additionally, follicle-dwelling bacteria, the patient's genetics, the skin microbiome, and therapeutic parts of diet are all important aspects of acne treatment [125]. One of the latest strategies that may be successful in treating acne is the use of medications that emit nitric oxide (NO). Highly powerful anti-inflammatory, antibacterial, and antioxidant properties are only a few of NO's various uses [126], comprising teratogenicity, skin and eye responses, changes in blood markers, and occasionally acne fulminans. By

considering combinations of drugs and modifying the dosage, it is crucial to concentrate on reducing these adverse effects. Patients' treatment perceptions will be enhanced as a result. One powerful vitamin A compound used to treat acne is isotretinoin (ISO). It may cause acne to go into persistent remission. Over the last 20 years, several attempts have been made to develop innovative therapeutic regimens primarily directed at getting rid of acne, even though it is known to have negative effects [75].

TABLE 2.1: List of current commercial products available in acne treatment [85]

Trade Name	**Active Compounds**	**Severity of Acne**	**Therapeutic Category**	**Manufacturing Company**
Differin	Adaplene retinoids	Mild to moderate	Topical gel form	Nestle pharmaceuticals
Benzamycin	Erythromycin	Mild to moderate	Topical gel form	Dermik laboratories
Winlevi	Androgen receptor inhibitors	Acne	Topical cream	Cassiopea pharmaceuticals
Solodyn	Minocycline tetracycline antibiotics	Acne	Oral	Teva pharmaceuticals
Absorica	Isotretinoin retenoids	Severe acne	Oral	Hoffmann roche
Vibramycin	Tetracyclines antimalarials	Acne	Oral	Pfizer pharmaceuticals
Accutane	Isotretinoin systemic	Severe acne	Oral	Roche pharmaceuticals

2.6 Natural Products in Acne Management

Due to growing resistant bacteria to antibiotics and adverse effects from traditional treatments, the use of natural medicines and herbal remedies in dermatological illnesses, especially acne vulgaris, has attracted a lot of scientific attention. Flavonoids, alkaloids, terpenoids, phenolic acids, and essential oils are examples of phytochemicals with biological activity that have shown antibacterial,

anti-inflammatory, antioxidant, and regulate sebum properties that address the complex pathophysiology of acne [127]. By preventing the replication of Staphylococcus aureus, *C. acnes* (previously *Propionibacterium acnes*), and the other bacteria linked to acne, plant-based antimicrobial substances are essential to the treatment of acne. Plant-based extracts including Azadirachta indica, Aloe vera, and Camellia sinensis, as well as essential oils like tea tree oil, thyme, clove, and rosemary, have significant bacteriostatic and bactericidal properties. These botanicals lower microbial colonization on the skin and minimize symptoms of inflammation, according to a number of in vitro and clinical investigations [128]. Natural substances have antibacterial properties as well as the ability to reduce inflammation linked to acne lesions. Green tea polyphenols, grape resveratrol, and turmeric curcumin have been shown to reduce inflammation, pain, and lesion frequency by inhibiting pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) and NF-κB activation. Compared to synthetic medications that only affect one route, these multiple targets activities are thought to be beneficial [129].

Phytochemicals' antioxidant qualities also help to treat acne since acne-causing acne production is linked to stress caused by oxidation and lipid peroxidation of sebum triglycerides. Antioxidants including vitamin C, quercetin, vitamin E and catechins reduce the formation of acne lesions by neutralizing free radicals and restoring the redox equilibrium in the skin microbial environment [130]. The anti- sebum and dermatological properties of botanicals have been emphasized in recent studies. Plant extracts such as Nigella sativa, Glycyrrhiza glabra, and Cinnamomum zeylanicum show inhibitory effects on 5-α-reductase activity, which reduces androgen-driven sebum production. Furthermore, azelaic acid, a naturally occurring dicarboxylic acid, and salicylic acid from Salix alba (willow bark) have keratolytic actions that aid in unclogging pores and encouraging skin rejuvenation [131]. Significantly, the combination of nanotechnology with natural products, including liposomes, polymeric nanoparticles, and nanoemulsions, has demonstrated improved plant chemical transport to the pilosebaceous cells and sebaceous glands. This offers promising substitutes for traditional topical therapies by enhancing stability, bioavailability, and absorption of natural anti-acne chemicals [132]

2.7 Plants Having Antiacne Potential

The search for ways to deal with skin breakouts continues to be a major creative effort in pharmaceutical and personal research projects [133].

The concept of the relationship between microscopic organisms and anti-infection agents is one of the many factors contributing to the growth of anti-infection opposition [134].

Thus, there are enough justifications to examine elective treatments that address and identify these problems. Restorative plants have been considered as choice treatments for infections in order to address anti-toxin resistance and the high expense of therapy. Several publications have shown the potential of using restoratively potent plant actives as an optional way to avoid the growth of microorganisms and provocative reactions [85]

2.7.1 Phenolic Compounds

Phenolic and polyphenolic substances act as the main components of most plant physiology because of their ability to act against *C. acnes* due to their anti microbial, anti inflammatory and anti inflammatory activities. Following table shows some herbs containing anti-acne polyphenolic compounds and their mode of action against acne [135].

TABLE 2.2: Some herbs composing anti-acne polyphenolic compounds [135]

Plants	**Common Name**	**Part of Plant Used**	**Properties**	**Active Compound**
Berberis vulgaris	Barberry	Roots and fruits	Anti-inflammatory	Flavonoids and anthocyanins
Aloe barbadensis	Aloe vera	Gel of leaf	Anti-acne, anti-inflammatory, antimicrobial	Emodin and aloin

Table 2.2 continued from previous page

Plants	**Common Name**	**Part of Plant Used**	**Properties**	**Active Compound**
Camellia sinesis	Green tea	Leaves of plant	Anti-micrbial, anti-inflammatory, anti-acne	EGCG,EC,ECG,EGC
Embilica officinalis	Amla	Mostly fruit of plant	Antioxidant, anti-inflammatory, antimicrobial	Gallic acid, methyl-gallate, corilagin, furosin
Lavendula stoechas	Lavender	Flowers of plant	Anti-microbial, anti-oxidant	Caffeic acid, luteolin, quercitin
Mangifera indica	Mangoes	Seed kernel	Antimicrobial, antioxidant	Gallic acid
Morus alba	White mulberry	Stem cortex	Anti-inflammatory, antimicrobial	Polyphenols
Olea europaea	Olives	Leaves of plant	Anti-acne, antimicrobial, anti inflammatory	Apigenin, flavonoids, luteolin
Rheum ribes	Rhubarb	Roots of plant	Antimicrobial, anti-inflammatory	Rhein, emodin
Zingiber officinale	Ginger	Rhizomes	Anti-acne, anti-inflammatory	Shogols and gingerols

2.7.2 Terpenoids and Steroids Compounds

Terpenoids are the considered as most diverse and variant group among secondary metabolites of pants. Due to their anti bacterial activity terpenoids plays vital role in acne treatment following table shows some herbs containing anti-acne steroids and terpenoids [135].

TABLE 2.3: Herbs containing terpenoids and steroids [135]

Plants	**Common Name**	**Part of Plant Used**	**Properties**	**Active Compound**
Eucalpytus globulus	Eucalyptus	Leaves	Antimicrobial, anti inflammatory, anti-liogenics	γ-terpinene

Table 2.3 continued from previous page

Plants	Common Name	Part of Plant Used	Properties	Active Compound
Boswellia serrata	Olibanum tree	Olio gum resins	Antimicrobial, antioxidant	Beta-boswelic acid, 3 - acetyl - beta - boswellic acid
Commiphora mukul	Guggul	Resins	Anti -oxidant, anti-inflammatory	Myrrhanol A, triterpenes, myrrhanone A
Lavendula stoechalus	Lavender	Flower	Anti-inflammatory, antimicrobial	Fenchone, camphor
Momordica charantia	Bitter melon	Fruit leaves	Anti-inflammatory	Nostocioone and its derivatives

2.8 *C. colocynthis* Bitter Apple: Medicinal Importance

A yearly herbaceous vine that belongs to the Cucurbitaceae family, *C. colocynthis*(L.) Schrad is found all over desert regions such as the Arabian Peninsula, Sahara, Sudan, and southern Asia (including Pakistan). It was transported to areas like Spain and Cyprus by ancient routes of trade. Its globular, bitter-tasting fruits, which usually contain 250 seeds per gourd, are used for whatever from topical application by nomads to consumption for a variety of diseases. In South Punjab, they are sometimes used with jaggery, however in Jordan, they are used as diuretics and abortive [136].

C. colocynthis has been used as a purgative, anti-inflammatory, antidiabetic, analgesic, hair promoting, abortion inducer, and antiepileptic agent in standard Iranian medicine. However, because of its limited therapeutic profile as well as associated risks, such as colic, diarrhea, hematochezia, nephrosis, and vomiting, herbalists have approached its use very carefully [137].

Phytochemical analyses of *C. colocynthis* discovered an extensive array of bioactive components. Depending on the plant part and extraction solvent used, these include alkaloids (such as quinoline derivatives), flavonoids, phenolic acids, coumarins,

steroids, volatile compounds, hydrocarbons, fatty acids, α-pinene, and thymol, as well as cucurbitacins (especially cucurbitacins A–L and specific glycosides like cucurbitacin E 2-O-β-D-glucopyranoside) [138].

2.8.1 Taxonomy and Distribution

C. colocynthis(L.) Schrad., sometimes referred to as bitter apple, or desert gourd, is a member of the Cucurbitaceae family, which is well-known for its members' significant industrial and therapeutic value. It is a herbaceous, spreading, perennial vine with deeply lobed leaves, hairy, rough stems, and single yellow blooms. The plant generates tiny, spherical fruits with a bitter flesh containing bioactive chemicals of medicinal significance. The fruits are green while they are young and become yellow when they ripen [139].

The accepted taxonomic hierarchy of *C. colocynthis* presented below:

Kingdom: Plantae
Clade: Tracheophytes → Angiosperms → Eudicots → Rosids
Order: Cucurbitales
Family: Cucurbitaceae
Genus: Citrullus
Species: *C. colocynthis*

Based on structural, cellular, and genetic markers, the species has been taxonomically separated from its near relatives, including Citrullus lanatus (watermelon). Its different classification within the genus is further strengthened by its unique phytochemical profile, especially the presence of cucurbitacins [140].

2.8.2 Geographical Distribution of *Citrullus colocynthis*

The xerophytic plant species *C. colocynthis* typically occurs in arid and semi-arid areas of the Old World. *C. colocynthis*is a significant medicinal resource for

traditional medical traditions like Unani and Ayurveda since it grows widely in the Thar Desert, Cholistan Desert, Sindh, Rajasthan, and Gujarat in South Asia, especially in Pakistan and India [141].

2.8.3 Traditional and Ethnomedicinal Uses of *Citrullus colocynthis*

C. colocynthis is used for its antibacterial, purgative, and anti-inflammatory qualities. Its fruit pulp and seeds were prepared and used as treatments for inflammatory disorders, ulcers, and skin problems [142].

This plant is highly regarded in Unani medicine for its detoxifying and blood-purifying properties, and it is mostly used to treat boils, acne outbreaks, and chronic skin conditions. It was thought that by controlling excessive heat and blood pollutants, its bitter properties would lessen skin problems[143].

Constipation, infections, rheumatism, and skin eruptions were all treated with colocynth fruit, according to Arabic and Middle Eastern folk medicine. Seed oil was used to relieve inflamed skin, while decoctions were frequently administered topically for wounds, dermatitis, and acne [144].

Poultices produced from *C. colocynthis* had been used as wound-healing and antiseptic remedies in North African and Middle Eastern populations, demonstrating their significance in dermatology[145].

South Asian ethnobotanical sources emphasize the plant's ability to heal respiratory disorders, diabetes, gastrointestinal problems, and skin diseases. Traditionally, colocynth extracts were used topically to alleviate skin inflammation, pustules, and comedones, hinting to its potential to treat acne today [146]

The ethnomedical record shows that *C. colocynthis* has long been linked to dermatological uses, especially for acne-like diseases, confirming its applicability as a potential option for modern phytotherapeutic and in silico research [147].

2.8.4 Pharmacological Activities of *C. colocynthis*

A wide range of medicinal uses, such as anti-inflammatory, antimicrobial, antioxidant, antidiabetic, anticancer, and immunomodulatory effects, have been reported for *C. colocynthis* fruit pulp and fractions. These effects are typically attributed to phenolics, flavonoids, cucurbitacins, and volatile components found in all solvent components. Numerous in vitro and in vivo studies show that *C. colocynthis* extracts have anti-allergic and analgesic qualities.

Experimental procedures (such as formalin tests and carrageenan-induced paw edema) demonstrated significant inhibition of inflammatory markers and pain responses following fruit extract application, demonstrating traditional uses for inflammatory illnesses and pointing to potential techniques associated with regulation of proinflammatory chemicals. Numerous studies have shown that *C. colocynthis* seed, the pulp, rind, and leaf extracts have antibacterial, antifungal, and antiplaque properties contrary to a variety of Gram-positive and Gram-negative pathogens (such as Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and oral pathogens). Time-sensitive bactericide impact measured inhibitory boundaries, and MIC/MBC readings for oil and solvent extracts were determined.

Evaluations like DPPH, FRAP, and total phenolic/flavonoid quantity calculations have been used to quantify the antioxidant potential of *C. colocynthis*; pulp and some accessions frequently exhibit high total phenolic content and free-radical scavenging capacity, suggesting that phenolic compounds are the main contributors to antioxidant activity and supporting antioxidative assertions in pharmacological examinations. Isolated cucurbitacins and other triterpenoids from *C. colocynthis* display antiproliferative and immunomodulatory effects in cellular models; cucurbitacin derivatives inhibited lymphocyte proliferation and showed cytotoxicity against cancer cell lines, with molecular docking and in-silico studies supporting interactions with cancer-related targets and highlighting these molecules as bioactive lead scaffolds.

Healing wounds, antidiabetic, pesticidal, and liver protection are among the other bioactivities that have been reported. However, scientific research also highlights

toxicity issues and a limited therapeutic index in conventional applications; recognized side effects, such as digestive distress and nephrotoxicity, call for careful dosage and additional toxicological profiling prior to clinical implementation [149].

2.8.5 Relevance of *C. colocynthis*in Acne Treatment

The widely recognized herbal remedy *Citrullus colocynthis*, sometimes identified as bitter apple, is a member of the Cucurbitaceae family and has long been used in Ayurvedic, Unani, and conventional medical practices for the management of a variety of infections and inflammatory diseases. Cucurbitacins, flavonoids, alkaloids, glycosides, saponins, and essential fatty acids are among its bioactive components; many of these have antibacterial, antioxidant, and anti-inflammatory qualities [149].

Cucurbitacins (E, B, and I), quercetin, isovitexin, and analogues of caffeic acid were all discovered in *C. colocynthis*, according to several phytochemical investigations. These substances have been shown to prevent the synthesis of inflammatory mediators such TNF-α, IL-6, and nitric oxide (NO), that are important components of inflammation linked to acne [150].

Extracts derived from *C. colocynthis* have been shown to have antibiotic action against a variety of pathogens, such as Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, and Klebsiella pneumoniae. Since Staphylococcus aureus and *C. acnes* are two of the main microorganisms linked to acne vulgaris, this procedure is very important for acne treatment. Strong zones of inhibition identical to that exist in traditional antibiotics have been demonstrated in vitro by ethanolic and methanolic extracts of seeds and fruits [151].

C. colocynthis high flavonoid and phenolic content is responsible for its antioxidant potential. Since oxidative stress increases lipid peroxidation in sebaceous secretions, which results in comedogenesis, antioxidants are crucial in the treatment of acne. Research has shown that the fruit's methanolic extracts considerably scavenge DPPH radicals and lower oxidative stress, indicating its potential to lessen the severity of acne [152].

Additionally, because certain of its constituents prevent lipogenesis and androgen-induced sebaceous gland activity, the herb has anti-sebum and keratolytic qualities. These activities help reduce excessive generation of sebum, which is a major contributing element to the pathophysiology of acne [153].

Recent developments in *C. colocynthis* extract compositions based on nanotechnology (such as nanoparticles, gels, and nanoemulsions) have demonstrated enhanced skin penetration, stability, and bioavailability, consequently augmenting its anti-acne efficacy. These dosage forms show promise as a direct delivery method for bioactive chemicals obtained from plants to pilosebaceous units [154].

When combined, *C. colocynthis* diverse pharmacological characteristics—which include antimicrobial, anti-inflammatory, antioxidant, and anti-sebum effects—make it a compelling option for the creation of cutting-edge anti-acne treatments, especially when investigated using computational docking and in silico techniques [155].

2.8.6 Bioactive Compounds of *C. colocynthis* with Anti-Acne Potential

A wide range of bioactive substances, including as cucurbitacins, flavonoids, alkaloids, fatty acids, and phenolic derivatives, are responsible for *C. colocynthis* anti-acne properties. These metabolites work in concert to reduce oxidative stress, control inflammatory responses, inhibit microbes, and regulate sebum [156].

TABLE 2.4: Bioactive compounds of *C. colocynthis*

Compound	**Phytochemical Class**	**Pharmacological Action**	**Relevance to Acne**
Cucurbitacin B,D,E	Triterpenoid	Anti-inflammatory, Antimicrobial, Antioxidant	Inhibits *C. acnes*, reduces cytokine expression (TNF-α, IL-6)
Flavonoids	Polyphenol	Anti-oxidant, anti inflammatory	Reduces ROS in acne lesions, prevents scarring

Table 2.4 continued from previous page

Compound	Phytochemical Class	Pharmacological Action	Relevance to Acne
Saponins	Glycoside	Antibacterial,biofilm disruption	Enhances microbial clearance, aids penetration of other agents
Phenolic acid	Phenolic compound	Anti-inflammatory, radical scavenging	Protects skin from oxidative stress and damage
Fatty acids	Lipids	Sebostatic effect, Skin barrier support	Improves skin healing
Alkaloids	Nitrogen containing bases	Antimicrobial, Enzyme inhibition	Disrupts bacterial function and inflammatory enzyme pathways
Tanins	Polyphenol	Astringent, Anti-inflammatory, Antioxidant	Tightens pore, reduces inflammation, and prevents bacterial growth
Terpenoids	Isoprenoid	Anti-inflammatory, Antimicrobial	Wound-heal, supports skin regeneration, reduces microbial burden

2.8.6.1 Cucurbitacins

The Cucurbitaceae family contains tetracyclic triterpenoids known as cucurbitacins. Cucurbitacins B, E, and I are the most noticeable in *C. colocynthis*. By suppressing JAK/STAT signaling, lowering pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), and preventing keratinocyte hyperproliferation—a key factor in the development of acne lesions—these substances have strong anti-inflammatory effects. Cucurbitacins also has antibacterial capabilities against Gram-positive bacteria, which may include *Cutibacterium acnes* [157].

2.8.6.2 Flavonoids

The fruit and seed extracts are rich in flavonoids, including variants of apigenin, isovitexin, and quercetin. Reactive oxygen species (ROS) produced in sebaceous

glands are neutralized by these molecules, which have a powerful antioxidant property. Additionally, quercetin reduces inflammatory lipid mediators that worsen acne lesions by inhibiting the lipoxygenase and cyclooxygenase pathways. Furthermore, flavonoids inhibit sebum production by modifying androgen receptor function [158].

2.8.6.3 Alkaloids

Colonynthine and colocynthenine, two alkaloids found in *C. colocynthis*, have significant antibacterial and anti-inflammatory qualities. They have beneficial effects versus S. aureus and *P. acnes* because they impair microbiological membrane functioning and protein production. Additionally, certain alkaloids decrease inflammation linked to acne by downregulating NF-κB signaling [159].

2.8.6.4 Fatty Acids

These seeds are high in palmitic acid, oleic acid, and linoleic acid. A recognized key element to the pathogenesis of acne is a lack of linoleic acid in sebaceous follicles. Linoleic acid supplementation improves keratinocyte differentiation, decreases comedone development, and restores skin barrier function. Additionally, the unsaturated fatty acids in the seed oil control lipid peroxidation in sebum and have bactericidal actions against bacteria linked to acne [160].

2.8.6.5 Phenolic Compounds

Essential components of *C. colocynthis* include phenolic acids such ferulic acid, gallic acid derivatives, and caffeic acid. These substances prevent comedogenesis which is caused by lipid peroxidation, scavenge free radicals, and lessen oxidative stress in sebaceous glands. Additionally, they support the plant's synergistic antibacterial and wound-healing properties, which are helpful in managing acne scars [161]. These bioactive substances work together to target many acne-related processes, *C. colocynthis* is a promising option for innovative phytotherapeutic

strategies. Its promise as an integrated anti-acne therapy is supported by their combined effects on oxidative stress, microbial development, inflammation, and sebum management [161].

2.8.7 Toxicity and Safety Concerns of *Citrullus colocynthis*

C. colocynthis also possess some poisonous qualities despite its great medicinal potential. This is mainly because it contains chemicals that are extremely bitter and irritating, particularly cucurbitacins. Despite being bioactive, excessive intake of these triterpenoids can result in significant gastrointestinal irritation, diarrhea, abdominal discomfort, and even harm to your intestinal mucosa [162].

The symptoms of colocynth poisoning, which can range from electrolyte imbalance and bloody diarrhea to multi-organ malfunction in extreme cases, have been documented in clinical case reports. Large dosages or incorrect usage of traditional treatments are usually linked to such outcomes [163]. High dosages of colocynth extracts have been shown in animal trials to cause hepatotoxicity, nephrotoxicity, and reproductive toxicity, raising questions about its limited therapeutic window [164]

However, controlled investigations indicate that when non-toxic fractions (such seed oil or aqueous extracts) are employed in place of entire fruit pulp, modest and well-regulated dosages of *C. colocynthis* extracts may have therapeutic benefits without causing considerable toxicity [165]. In order to reduce toxicity, recent pharmacological assessments stress the significance of dose uniformity, extraction procedures, and formulation strategies. To lessen the systemic toxicity of cucurbitacins while maintaining their pharmacological potential, contemporary strategies including targeted drug delivery systems and nanoparticle encapsulation are being researched [166]. Therefore, even though *C. colocynthis* has significant therapeutic potential, especially in dermatology and anti-acne research, its toxicity profile necessitates cautious consideration. Its smooth translation from

ethnomedicine to contemporary pharmacology requires standardized formulations and a thorough safety evaluation [167].

2.9 Molecular Docking

Molecular docking is a computerized methodology that provides information on binding affinities and interaction patterns by predicting the preferred orientation of a ligand when attached to a target protein [168].

In contemporary drug development and natural product research, it is extensively used, especially for the identification of bioactive chemicals from medicinal plants. Researchers may assess whether a chemical can fit into a receptor's active site and gauge the intensity of its binding by using both structural and energetic information provided by docking simulations [169].

Docking's primary focus is to mimic the "lock-and-key" or "induced fit" model of recognition of molecules. The ligand functions as a key, the protein as a receptor (lock), and various ligand conformations and orientations (poses) inside the receptor binding region are investigated using computer methods [170].

Binding energy is subsequently determined using scoring algorithms; stronger and more advantageous interactions are often indicated by higher negatives scores.[171].

For phytochemicals with many different biological functions, such as alkaloids, flavonoids, and terpenoids, docking is very useful. concentrating on compounds derived from plants with high binding affinities against disease-related proteins by in silico docking helps researchers to confirm these compounds through experimental testing. For example, major inhibitory potentials have been shown when natural bioactive substances are docked against enzymes such as tyrosinase, lipase, or kinases, highlighting their medicinal benefits.

Studies on docking often use several software tools, including AutoDock, PyRx, GOLD, and Glide. In order to predict the best binding positions, these computer systems use a variety of search techniques, including as Monte Carlo simulations,

genetic algorithms, and Lamarckian genetic algorithms. The kind of receptor, ligand flexibility, and computing resources all influence the tool selection. PyRx is frequently employed for natural chemical screening due to its user-friendly interface and connectivity with AutoDock Vina .In acne investigation, docking is also necessary for examining ligands from medicinal plants against acne-related targets such keratin proteins, inflammatory cytokine regulators, and *Cutibacterium acnes* lipase. Docking offers insights into possible inhibitory processes and medicinal uses by modeling ligand-receptor interactions.

Molecular docking also has disadvantages despite its benefits. Entropy, solvation effects, and protein flexibility are frequently simplified too much which can lower prediction accuracy. As a result, in vitro tests and molecular dynamics (MD) simulations are often used in conjunction with docking experiments to confirm the expected results [171].

2.10 Comparative Perspective with Other Anti-Acne Medicinal Plants

The anti-inflammatory, antibacterial, and antioxidant properties of tea tree scientifically known as Melaleuca alternifolia include effectiveness against *Cutibacterium acnes* and lesion-count decreases in topical investigations, according to reviews and clinical data [172].

The regulation of sebum and anti-inflammatory effects have been demonstrated by green tea (Camellia sinensis, EGCG); a single-blind study of topical EGCG showed a major decrease of inflammatory and non-inflammatory lesions over 8 weeks along with favorable tolerance, proving its role as a non-antibiotic option [173].

A random, double-blind, placebo-operated research study of a topical Nigella sativa formulation in mild-to-moderate acne displayed major improvements compared to placebo, suggesting anti-inflammatory and antimicrobial contributions relevant to acne care. Nigella sativa, also known as black seed, exhibits clinical

promise [174]. The anti-inflammatory flavonoids and triterpenes found in licorice (Glycyrrhiza spp.) reduce TNF, MMPs, PGE_2, and oxidative mediators, providing a mechanism for the use of licorice as an adjuvant treatment for acne in areas where inflammatory activity is predominant [175].

Although it does not have direct antibacterial suppression towards *C. acnes*, witch hazel (Hamamelis virginiana) has significant anti-inflammatory effects on *C. acnes*-induced responses (e.g., cytokine modulation), suggesting utility primarily as a calming/adjunct ingredient rather than as a primary antimicrobial acne therapy [176].

Neem exhibits multiple therapeutic potential; systems-level investigations that combine molecular modeling and network pharmacy identify pathways related to acne etiology that are antibacterial, anti-inflammatory, and regulate sebum production, prompting more translational assessment [177].

Further mechanistic research confirms the anti-inflammatory significance of limonoids like gedunin as inhibitors of *C. acnes*-triggered NF-κB/NLRP3 signaling in preclinical animals [179].

When employed in non-drug combination topical regimens, aloe vera can be an additional topical component. Clinical and mechanistic evidence show anti - inflammatory, healing wounds, and barrier-supporting benefits, including improvement in acne [179]. A wider natural-product space for antibacterial leads vs acne is shown by the in vitro activity of oil-rich essential botanicals other than tea tree against *C. acnes*, with strength in some cases equivalent to conventional topical antibiotic [181]. Nanoemulsion techniques and combinations (such as with adapalene) to improve efficacy and acceptability in clinical settings are also described in reviews of therapeutic use of essential oils [180]

Positioning in relation to *Citrullus colocynthis*: when compared to plants with human clinical data or focused mechanistic studies in acne, such as tea tree, green tea, and Nigella sativa, C. With minimal clinical data related to acne, colocynthis now has more ethnomedical record and more general antimicrobial/dermatologic

results, indicating the importance for targeted molecular and translational research [182].

2.11 Research Gap in Existing Literature

There are still a number of gaps in the body of knowledge on medicinal plants that may be able to prevent acne. The antibacterial, antioxidant, and anti-inflammatory properties of medicinal plants have become the subject of several investigations, but only a few of them have been thoroughly examined against *Cutibacterium acnes* using sophisticated in vitro and in silico techniques [183]. Additionally, despite *Citrullus colocynthis*'s notable antibacterial, antifungal, and wound-healing qualities, the opportunity for treating acne is still untapped [184]. The majority of studies concentrate on crude extracts, which lack compound-specific insights, which is another drawback. The translation of discoveries into pharmaceutical development is limited by the lack of thorough bioactive compound profiling and molecular docking confirmation against acne-related protein targets [185]. The relative efficacy of *C. colocynthis* is also little recognized due to lack of comparative trials involving other anti-acne medicinal plants [186].

Furthermore, whereas pharmacokinetic estimates and in silico ADMET have become crucial techniques for drug development, their application to *C. colocynthis* bioactive molecules has not been systematic [187]. This gap makes it more difficult to find lead compounds with practical prospects that are both safe and effective. In order to officially recognize *C. colocynthis* as a viable option for anti-acne therapy, the literature indicates a critical need for integrative techniques including phytochemical characterisation, molecular docking, ADMET profiling, and comparative effectiveness investigations.

Chapter 3

Methodology

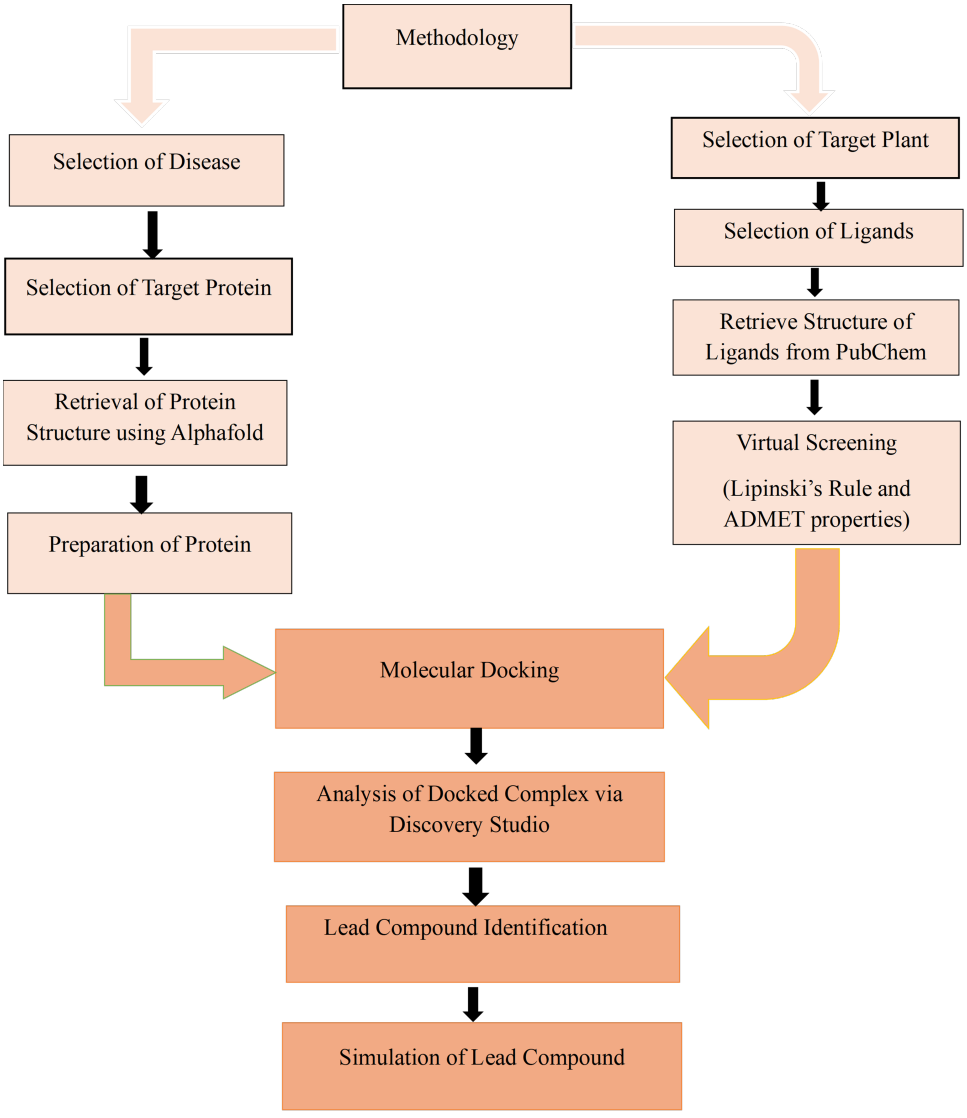


FIGURE 3.1: Flow Chart for Methodology

3.1 Databases and Tools Used

3.1.1 Databases

The study use various databases for literature retrieval and analysis of data which includes following.

3.1.1.1 PubMed

PubMed is freely accessible website which is regulated by NCBI (National Centre for Biotechnology Information) and (NLM) National Library of Medicine of US, (pubmed.ncbi.nlm.nih.gov). Medical Subjects Headings are employed by this website to categorize articles related to subjects. It provides access to variety of articles, research papers and publications in various subjects such as health, medicine and life sciences. PubMed is easily accessible to anyone with internet.

In order to find peer-reviewed research papers about keratin proteins, antimicrobial plant substances, acne development, and molecular docking investigations, this study utilized PubMed as their primary literature retrieval resource.

Boolean operators were employed to apply certain keyword combination, which include "acne vulgaris AND keratin 16," "Citrullus colocynthis phytochemicals," and "plant ligands molecular docking." Target selection, ligand justification, and methodology design were all assisted by the selection, screening, and citation of English-language publications in essential scientific domains.

3.1.1.2 Google Scholar

It is a search engine for the internet developed to find academic books, theses, conference papers, articles, and patents. It delivers full-text article links, citation tracking, author biographies, notifications, complex search filters, Google Scholar metrics, and extensive coverage across several fields. (scholar.google.com).

As an extra educational search engine, Google Scholar was employed to identify full-content articles, papers from conferences, theses, and other scholarly resources that weren't present in PubMed's database. It was particularly beneficial for tracking citations, finding frequently cited research, and finding supporting references for studies on protein-ligand interactions, docking tools, and ADMET prediction.

3.1.1.3 AlphaFold Database

AlphaFold is a popular computational database that makes use of methods based on artificial intelligence for precisely predicting the three-dimensional structures of proteins [188]. AlphaFold, built by DeepMind, models protein folding from the amino acid sequences using profound learning and neural networks with topologies trained on scientifically established protein structures [189].

Millions of predicted protein structures that include a substantial amount of known protein sequences from many animals are accessible for free through the AlphaFold Protein Structure Database [190].

AlphaFold is a useful tool for structural biology and drug discovery research since these predicted structures have frequently shown accuracy like experimentally resolved models, especially for globular proteins [18, 188].

The database makes it easier for researchers to integrate protein structures with molecular docking and simulation studies by allowing them to be seen, analysed, and downloaded in common formats [190].

AlphaFold has become a vital tool for researching protein structure–function connections in circumstances when experimental findings are not readily available because of its dependability, accessibility, and wide coverage [189, 191].

Since the chosen target protein's experimentally identified structure was not available in the Protein Data Bank, the three-dimensional structure was retrieved using the AlphaFold Protein Structure Database. The expected model was ready for molecular docking assessment after being downloaded in PDB format. The

structural visualization, active-site identification, and interaction assessment required to conduct ligand binding simulations have been rendered possible by this structure.

3.1.1.4 PubChem

A freely available resource known as Pub Chem gives extensive knowledge about molecules, including their characteristics, synthesis, patents, drug labelling, spectrum data, research in clinical trials, and molecular structures and traits (pubchem.ncbi.nlm.nih.gov/).

To obtain the molecular information and chemical structures of a few chosen phytocompounds from Citrullus colocynthis, PubChem was utilized. The ligand was constructed using canonical SMILES, 2D structures, molecular weight, and physical and chemical characteristics prior docking and ADMET prediction analysis.

3.1.1.5 PkCSM

This (pkCSM) is an approach that generates predictive models of important ADMET characteristics for drug development using graph-based signatures. It is a free to use web server offering an integrated platform for fast evaluating pharmacokinetic and toxicological features while not storing any data supplied to it (http://structure.bioc.cam.ac.uk/pkcsm) [192].

The pharmacokinetic and toxicity characteristics of certain ligands were estimated using pkCSM. For evaluation of ADMET parameters, such as absorption, distribution, metabolism, excretion, and toxicity endpoints, SMILES formats of each compound have been provided. In order to assess the safety profiles and drug-like characteristics of potential chemicals, the produced findings were studied.

3.1.2 Tools

Following tools are used in this study.

3.1.2.1 BIOVIA Discovery Studio Visualizer

Proteins and other minor data could be seen, shared, and analyzed using this feature-rich, free molecular modeling program. Molecular visualizing is an essential tool for modeling study analysis and communication. In the current study, this tool was employed for the analysis and visualization of proteins and ligands. (www.3ds.com/products/biovia/visualization).

Following docking, protein-ligand complexes were viewed and analyzed using BIOVIA Discovery Studio Visualizer. It enabled the ability to examine active-site residues, hydrogen bonds, hydrophobic interactions, and binding conformations. For the purpose to analyze and illustrate the docking results, the program was additionally used to create high-resolution structural pictures and 2D interaction representations.

3.1.2.2 PyRx

PyRx is a virtual testing tool for computerized drug creation that examines a wide range of chemicals in relation to therapeutic targets. It help users at every stage, from data preparation to complete analysis. Its user-friendly design makes it a useful tool for drug discovery research. PyRx makes sense for structure-based drug design since it offers docking tools, spreadsheet-style functionality, and powerful presentation abilities. This program performs molecular docking utilizing many ligands and shows each ligand's best region for binding to the protein. The outcomes are assessed quantitatively by looking at the scoring algorithms and based on the assessment of the ligand location. When docking is completed, the greatest binding energy is see (https://pyrx.sourceforge.io/).

3.2 Selection of Disease

Acne vulgaris is one of the most dominant and severe inflammatory diseases that mostly occur in pilosebaceous units, both adolescents and adults are affected

throughout the world [193].

It mainly affects face, chest and even back often resulting from interaction between hyperkeratinization of follicles, too much sebum production and infection by Cucurbacterium acne and inflammation [2].

Even acne is not that critical, it profoundly affects the quality of life and is mostly linked with patient's psychological situations because patients might face anxiety, depression, and reduced self-worth [195].

Recent research has highlighted the contribution of dysregulation of immune system, oxidative stress, imbalance of hormones, and microbial resistance in the development of acne [196].

Moreover, the persistent utilization of standard therapeutic strategies such as antibiotics and retinoids results in worst effects and antibiotic-resistant strains are formed, emphasizing the need for safe and more efficient alternative treatments[193, 196].

Therefore, acne was selected as the disease of interest to gain intense view into its multifactorial pathogenesis and to explore new therapeutic strategies which targets inflammation, microbial colonization, and oxidative damage [194, 195].

3.3 Selection of Protein

Protein that is linked to development of acne was identified through comprehensive literature review. Research articles from PubMed which contain information about acne linked proteins were examined.

KRT16 (KRT16)was selected as target protein because it plays important role in maintaining skin barrier function and inflammatory responses [196]

3.3.1 Reasons for Selecting the KRT 16 Protein as a Drug Target

3.3.1.1 Overexpression in Hyperproliferative Epidermal Conditions

KRT16 is highly upregulated in hyperproliferative epidermal disorders and stress-induced skin conditions, where it contributes to abnormal keratinocyte activation and epidermal remodeling [197, 198].

3.3.1.2 Involvement in Follicular Hyperkeratinization

KRT16 plays a crucial role in keratin filament reorganization and keratinocyte differentiation, processes that are directly linked to follicular hyperkeratinization and comedone formation in acne pathogenesis [199].

3.3.1.3 Induction by Inflammatory Stimuli

Research indicates that KRT16 expression is strongly induced by inflammatory cytokines and mechanical stress, associating it with inflammation-driven skin disorders, including acne-related lesions [198, 200]

3.3.1.4 Relevance as a Therapeutic and Molecular Docking Target

Due to its consistent overexpression in diseased epidermis and its involvement in pathological keratinization, KRT16 is considered a promising molecular target for therapeutic intervention and in-silico inhibitor screening studies [197, 199].

3.4 Retrieval of Target Protein

The PubMeds IDs of papers containing information related to KRT16 protein were used as literature review. The predicted structure of human KRT16 was

downloaded from Alpha fold (share.google/RZ0RVYF84LFHr9yjI) in PDB format for further analysis and use in molecular docking.

3.5 Visualization of Protein and Ligands

The selected target protein, i.e. KRT16 and all ligand molecules were visualized using Discovery studio software.

3.6 Cleaning of Protein

Discovery studio visualizer was used for cleaning of protein. This step was done to clean the protein for further molecular docking. Normally, it involves removing water molecules, ligands or any other unnecessary compounds present in protein.

However, the downloaded protein structure was already clean having no water molecules or ligands bound to it. So protein was ready to use in further docking analysis.

3.7 Selection of Target Plant

C. colocynthis, commonly known as bitter apple, is a medicinal plant widely distributed in arid and semi-arid regions and has been extensively used in traditional medicine for the treatment of various inflammatory and infectious diseases [201].

The plant is rich in diverse bioactive compounds, including cucurbitacins, flavonoids, alkaloids, phenolics, and glycosides, which are known to exhibit strong antimicrobial, anti-inflammatory, antioxidant, and anti-acne properties [202].

Recent pharmacological studies have demonstrated that extracts and isolated compounds from *C. colocynthis* show significant inhibitory activity against acne-associated bacteria, particularly *Cutibacterium acnes*, along with modulation of

inflammatory mediators involved in acne pathogenesis [203]. Additionally, the presence of antioxidant constituents helps in reducing oxidative stress, which plays a crucial role in the initiation and progression of acne lesions [204].

Due to its broad therapeutic potential, natural origin, and comparatively lower risk of adverse effects, *C. colocynthis* was selected as the target plant to explore its bioactive compounds as potential alternative therapeutic agents for acne management [201, 203].

3.8 Selection of Ligands

Through an extensive literature review various phytochemical compounds present in *C.colocynthis*(bitter apple) were studied for their medicinal properties. Among these, the suitable 25 compounds showing potential antimicrobial, anti - inflammatory, and antioxidant activities as reported were selected for further analysis. The selected compounds were retrieved from database PubChem share.google/iOy3AF which provides chemical information in standard formats. All compounds were downloaded in Structure Data File (SDF) format for use in molecular docking studies. The description of these compounds with their PubChem IDs, are mentioned in the table below.

TABLE 3.1: Names and phytochemical families of chemical compounds

Sr no.	Compound Name	Phytochemical Family	Ref.
1.	Cucurbitacin E.	Triterpenoids	[205]
2.	Cucurbitacin B.	Triterpenoids	[205]
3.	Cucurbitacin L	Triterpenoids	[205]
4.	Cucurbitacin I.	Triterpenoids	[205]
5.	Cucurbitacin D.	Triterpenoids	[205]
6.	Quercetin.	Flavonoids	[205]
7.	Kaempferol.	Flavonoids	[205]
8.	Isoschaftoside.	Flavonoids	[205]
9.	Apigenin.	Flavonoids	[205]
10.	Luteolin.	Flavonoids	[205]
11.	Ferulic acid.	Phenolic acid	[205]

Table 3.1 continued from previous page

Sr no.	Compound Name	Phytochemical Family	Ref.
12.	Gallic acid.	Phenolic acid	[205]
13.	Vanillic acid.	Phenolic acid	[205]
14.	Caffeic acid.	Phenolic acid	[205]
15.	Chlorogenic acid.	Phenolic acid	[205]
16.	P-Coumaric acid.	Phenolic acid	[205]
17.	Catechin.	Flavonoids	[205]
18.	Epicatechin.	Flavonoids	[205]
19.	B-sitosterol.	Phytosterol	[205]
20.	Stigmasterol.	Phytosterol	[205]
21.	Campesterol.	Phytosterol	[205]
22.	Linoleic acid.	Fatty acid	[205]
23.	Palmitic acid.	Fatty acid	[205]
24.	Alpha-tocopherol	Vitamin	[205]
25.	Citrulline.	Amino acid	[205]

3.9 Virtual Screening

3.9.1 Lipinski Rule

To evaluate the drug-like nature of the selected phytochemicals, Lipinski's rule of five was applied. This guideline helps to predict whether a compound is likely to be a suitable candidate for oral medication or not. According to this rule, a promising drug molecule should generally meet the certain conditions :

Lipinski's rule of five refers to a set of criteria that suggests an orally active drug should not violate more than one of the following:

TABLE 3.2: Lipinski's parameters

Sr No.	Parameters	Standards
1.	Hydrogen bond acceptors	≤ 10
2.	Hydrogen bond donors	≤ 5
3.	Molecular weight	≤ 500 g/mol
4.	LogP	<5

Table 3.2 continued from previous page

Sr No.	Parameters	Standards
5.	Rotatable bonds	<10

PkCSM (https: //omictools.com/pkcsm-tool) is an online accessible tool that helps in determining the above-mentioned rules for compounds.

3.9.2 ADMET Properties

The ADMET (Absorption, Distribution, Metabolism , Excretion and Toxicity), properties of the selected ligands were predicted to evaluate their pharmacokinetic and safety profiles using pkCSM server. The pkCSM tool predicts these properties by providing information for drug-design , blood-brain barrier permeability , cytochrome p450 interactions [9].

This analysis helped in identifying compounds with good absorption , proper distribution in the body, favorable metabolism , efficient excretion and low toxicity. Compounds that passed both lipinski's rules and ADMET screening were prioritized for further interaction analysis with target protein.

3.10 Molecular Docking

Following the preparation of KRT16 (KRT16) protein, its structure was saved in PDB format which is compatible with PyRx software. The selected phytochemical ligands from *C.colocynthis* were also prepared by converting their SDF files, downloaded from PubChem, into PDB format using PyRx.In PyRx, the protein structure was uploaded and converted into a macromolecule file format (pdbqt) required for docking. Using Open Babel tool integrated within PyRx, all ligands were loaded, their energies were minimized and they were converted into pdbqt format as well. Next, the Vina Wizard option in PyRx was used to set up the docking process. The protein and ligands were selected and grid box dimensions were adjusted using the maximize option to ensure that protein was fully covered.

Finally, molecular docking was performed using Autodock Vina within PyRx. The docking results were recorded, focusing on the binding affinities of each ligand with the target protein. The best-scoring ligands were selected for further interaction analysis.

3.11 Analysis and Visualization of Protein Ligand Interaction

The results obtained from molecular docking were further analyzed using Discovery Studio Visualizer. This software was used to examine the detailed interaction between KRT16 and the docked ligands.

It provided clear information on the types of bonds formed such as hydrogen bonds, hydrophobic bonds, and electrostatic bonds, along with specific amino acids residues involved in binding.

In addition to interaction details, Discovery Studio also produced high-quality 2D and 3D visual representations of the protein-ligands complexes, making it easier to interpret and present the docking results.

3.12 Lead Compound Identification

Based on the results from lipinski'RO5 and ADMET screening ,the most promising compounds from bitter apple were shortlisted as lead compounds. Lead compounds are those which satisfied all properties.

Results from pkCSM server alongwith evidence from literature review supported therapeutic properties of these compunds in decreasing inflammation, and microbial activity as these are the key players in acne development.

3.13 Simulations

Applying Schrödinger program, the MD simulation of the designated lead compounds was carried out to determine the protein ligand complex's conformation flexibility. For 200 nanoseconds, the MD simulations were run at (NPT ensemble, 310 K, 1 atm).

Na^+ and Cl^- ions at a near-physiological salt concentration (∼51 mM) were used to neutralize the system after it had been soluble in a water box that contained about 20,093 water molecules. The simulated interaction diagram module was used to obtain the Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) plots in a format called PNG for more analysis.

3.13.1 Tools Used in Simulations

3.13.1.1 Schrodinger Molecular Modelling Suite

For structural-based drug discovery and biological molecules study, the Schrödinger molecular modelling software is a popular computing platform that combines docking of molecules, molecular dynamics simulations, and free-energy calculations [206]. A thorough examination of structural stability, conformational shifts, and intermolecular relationships over time is made possible by the software's atomistic-level simulations of protein–ligand complexes under physiologically realistic settings [207]. To be able to correctly simulate protein dynamics, solvent effects, and binding ligand behaviour, Schrödinger's simulation algorithms use complicated force field-based and verified procedures, that increases the accuracy of in silico predictions [208]. By analysing the stability over time of ligand–protein associations and identifying important residues that contribute to binding, molecular dynamics simulations completed utilizing Schrödinger techniques are very useful for confirming docking results [209]. This system continues to be frequently utilized in academic and pharmaceutical research to investigate mechanistic insights,

binding energetics, and protein flexibility that are not available with static structural analysis alone [210].

Chapter 4

Results

4.1 Selection and Preparation of Protein

4.1.1 Structure of Protein

For the present study, the human KRT16 protein was selected as the target due to its crucial role in acne pathology and its relevance to skin physiology. Since the experimentally resolved structure was not available, the predicted three dimensional structure of KRT16 was retrieved from the alphafold protein structure database. The protein model was obtained using its unique alphafold ID (AF-08779), DOI (https://alphafold.ebi.ac.uk/).

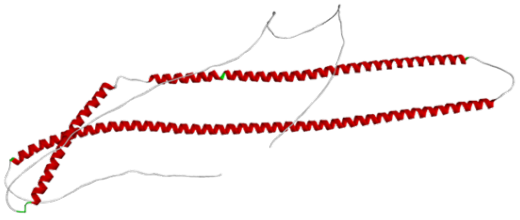


FIGURE 4.1: Structure of KRT16 Protein

The KRT16 genes codes for the human type I keratin protein KRT16, that is a member of the acidic keratin subgroup of intermediate filament proteins [211].

The protein, containing about 473 amino acids, displays the typical keratin construction, with non-helical N-terminal head and C-terminal tail segments on each side of a central α-helical rod domain [212]. All intermediary filament keratins share the intact portions 1A, 1B, 2A, and 2B of the rod domain, which can be distinguished by linker regions [213]. According to sequence analysis, the rod domain contains an extensive concentration of α-helical residues, which supports the structural progression of coiled-coil dimer molecules[214]. In human KRT16 (UniProt ID: P08779), the AlphaFold Protein Structure Databases provides a strong-confidence predicted 3-dimensional model with exceptionally high confidence evaluations within the central rod domain [215]. The N-terminal head and C-terminal tail sections exhibit decreased structural confidence according to AlphaFold predictions, which is typical with the fundamentally disorganized character of keratin proteins [216].

4.1.2 Cleaning of Protein

Generally, protein structures obtained from databases are purified using tools like discovery studio to remove heteroatoms, ligands or water molecules that may interfere with docking studies. However, the KRT16 structure retrieved from AlphaFold was already in clean form without any attached ligands or extra molecules therefore no additional purification was required in this study.

4.1.3 Ligands Selection and Preparation

PubChem is a freely accessible chemistry resource that provides comprehensive information on the structural physiochemical and biological properties of small molecules. The database maintained by the National Institute of health (NIH) is widely used for retrieving data on natural products, synthetic compounds and bioactive molecules relevant to drug discovery.

Literature review revealed 25 compounds and their respective structures were downloaded from PubChem in SDF format. Table 4.1 shows molecular weight,

formula and structures of all ligands. These ligands were converted into PDB format and visualized using Discovery Studio.

TABLE 4.1: Ligands with their 3D structures retrieved from Pubchem

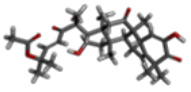
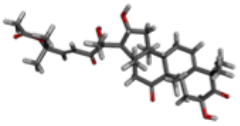
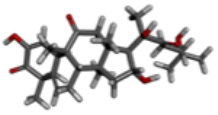
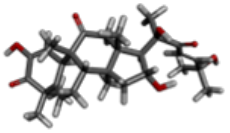
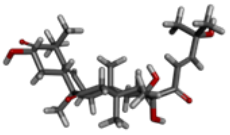
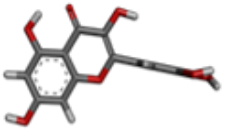
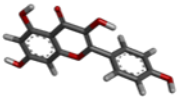
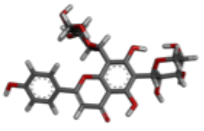
Sr no.	**Ligands**	**Molecular Formula**	**Molecular Weight**	**PubChem ID**	**3D Structures**
1.	Cucurbitacin E.	$C_{32}H_{44}O_8$	556.7	5281319	
2.	Cucurbitacin B.	$C_{32}H_{46}O_8$	558.71	5281316	
3.	Cucurbitacin L	$C_{30}H_{44}O_7$	540.69	441820	
4.	Cucurbitacin I	$C_{30}H_{42}O_7$	556.68	5281321	
5.	Cucurbitacin D.	$C_{30}H_{44}O$	514.65	5281318	
6.	Quercetin.	$C_{15}H_{10}O_7$	302.24	5280343	
7.	Kaempferol.	$C_{15}H_{10}O_6$	286.24	5280863	
8.	Isoschaftoside.	$C_{26}H_{28}O_{14}$	564.49	3084995	

Table 4.1 continued from previous page

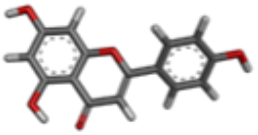
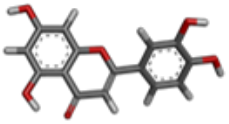
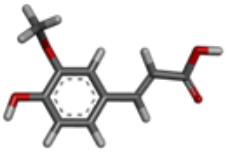
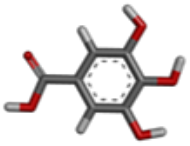
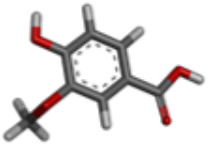
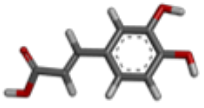
Sr no.	Ligands	Molecular Formula	Molecular Weight	PubChem ID	3D Structures
9.	Apigenin.	$C_{15}H_{10}O_5$	270.24	5280443	
10.	Luteolin.	$C_{15}H_{10}O_6$	286.24	5280445	
11.	Ferulic acid.	$C_{10}H_{10}O_4$	194.18	445858	
12.	Gallic acid.	$C_7H_6O_5$	170.12	370	
13.	Vanillic acid.	$C_8H_8O_4$	168.15	8468	
14.	Caffeic acid.	$C_9H_8O_4$	180.16	689043	

Table 4.1 continued from previous page

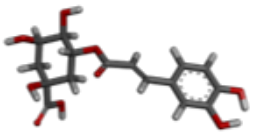
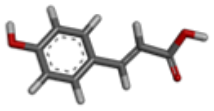
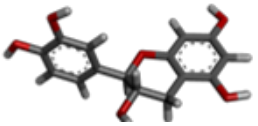
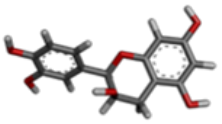
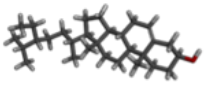
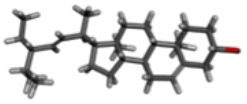
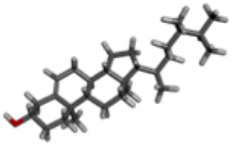
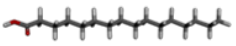
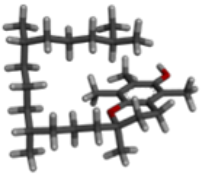
Sr no.	Ligands	Molecular Formula	Molecular Weight	PubChem ID	3D Structures
15.	Chlorogenic acid.	$C_{16}H_{18}O_9$	354.31	1794427	
16.	P-Coumaric acid.	$C_9H_8O_3$	164.16	637542	
17.	Catechin.	$C_{15}H_{14}O_6$	290.27	9064	
18.	Epicatechin.	$C_{15}H_{14}O_6$	290.27	72276	
19.	B-sitosterol.	$C_{29}H_{50}O$	414.71	222284	
20.	Stigmasterol.	$C_{29}H_{48}O$	412.69	5280794	
21.	Campesterol.	$C_{28}H_{48}O$	400.68	173183	
22.	Linoleic acid.	$C_{18}H_{32}O_2$	280.45	5280450	

Table 4.1 continued from previous page

Sr no.	Ligands	Molecular Formula	Molecular Weight	PubChem ID	3D Structures
23.	Palmitic acid.	$C_{16}H_{32}O_2$	256.42	985	
24.	Alpha-tocopherol	$C_{29}H_{50}O_2$	430.71	14985	

4.2 Screening of Bioactive Compounds for Lead Compound Selection

4.2.0.1 Virtual Screening through Lipinski's Rule of Five

To narrow down promising ligands for further study, Lipinski's Rule of Five was employed as an initial virtual screening filter. According to this rule a compound is more likely to be orally bioavailable if it satisfies at least three of the following conditions: molecular weight $\leq$ 500 Da, no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, and octanol-water partition coefficient (LogP) $\leq$ 5 [217].

According to table 4.2 Lipinski's rule of five was employed to evaluate the drug-likeness of a total of 25 selected ligands. The results revealed that 14 ligands obey all the parameters of Lipinski's rule, which indicates the good potential for oral drug usage. Eleven ligands violate only single rule, which does not actually limit their drug-like behavior, as compounds with one violation can still be considered acceptable. In contrast, only one of total 25 ligands, i.e Isochaftoside, violated three Lipinski criteria and was therefore categorized as unsuitable for direct drug use. Overall, these findings suggest that most of the selected ligands possess

favorable drug-likeness properties, with only one compound being excluded based on Lipinski's rule analysis.

Only those ligands that met these criteria were carried forward into docking experiments.

TABLE 4.2: Details of Lipinskin rule for 25 Selected Phytochemicals

Sr no.	**Ligands**	**Mol weight g/mol ≤500**	**LogP ≤5**	**Rotatable bonds**	**HBA ≤10**	**HBD ≤5**	**Yes/ No**
1.	Cucurbitacin E	556.696	4.1902	5	8	3	YES
2.	Cucurbitacin B	558.712	3.4993	5	8	3	YES
3.	Cucurbitacin L	516.675	3.8434	5	7	4	YES
4.	Cucurbitacin D	516.675	2.9285	4	7	4	YES
5.	Cucurbitacin I	514.659	3.6194	4	7	4	YES
6.	Quercitin	302.238	1.988	1	7	5	YES
7.	kaempferol	286.239	2.2824	1	6	4	YES
8.	Isochaftoside	564.496	-1.7543	4	14	10	NO
9.	Apigenin	270.24	2.5768	1	5	3	YES
10.	Luteolin	286.239	2.2824	1	6	4	YES
11.	Ferulic acid	194.186	1.4986	3	3	2	YES
12.	Gallic acid	170.12	0.5016	1	4	4	YES
13.	Vanillic acid	168.148	1.099	2	3	2	YES
14.	Caffeic acid	180.159	1.1956	2	3	3	YES
15.	Chlorogenic acid	354.311	-0.6459	4	8	6	YES
16.	P-Coumaric acid	164.16	1.49	2	2	2	YES
17.	Catechin	290.271	1.5461	1	6	5	YES
18.	Epicatechin	290.271	1.5461	1	6	5	YES
19.	B-Sitosterol	414.718	8.024	6	1	1	YES
20.	Stigmasterol	412.702	7.8008	5	1	1	YES
21.	Campestrol	400.691	7.6347	5	1	1	YES
22.	Linoleic acid	280.452	5.8845	14	1	1	YES
23.	Palmitic acid	256.43	5.5523	14	1	1	YES
24.	Alpha-tocopherol	430.717	8.84026	12	2	1	YES
25.	Phytol	296.539	6.3641	13	1	1	YES

4.2.1 ADMET Properties

4.2.1.1 Pharmacokinetics

Pharmacokinetics (Pk) explains the journey of a drug through the body over time, including how much it is absorbed in the body, mechanism of distribution and metabolization, and excretion which is commonly abbreviated as ADMET. It also considers how formulation, route of administration, and individual patient characteristics affect the drug concentration in plasma and tissues. Understanding PK is essential for determining dosage schedule that maximizes therapeutic effects while minimizing toxic effects [218].

4.2.1.2 Absorption

Absorption is the process by which a drug travels from its administration site to the systemic circulation, and it governs how much and how fast the drug becomes available in plasma. Factors such as solubility, permeability, formulation and physiological environment affect the rate and extent of absorption of drug. According to recent research, optimizing absorption is very critical because even highly active compounds may fail clinically if absorption is low [220].

According to table 4.3 following the intestinal absorption values, all the chemicals were categorized into three groups high absorbers (which shows greater than 80% absorption values), medium absorbers (which shows 30%-79% absorption values) and low absorbers (which shows less than 30% absorption values). Most compounds have good to excellent absorption (greater than 80%). Exceptions are Gallic acid (43%), chlorogenic acid (36%) and Isochaftoside (45%) which shows poor absorption due to their high polarity.

Following the water solubility values (Log S) , the interpretation shows scale from 0 (very soluble) to -10 (insoluble). Most drugs claim for $>$ -4. Many flavonoids, e.g Quercitin and Luteolin, are in acceptable range (-2 to -4). The Cucurbitacins, Sterols, Fatty acids (Linoleic, Palmitic, Phytol) have very poor solubility($<$ -5) which is major formulations challenge.

TABLE 4.3: Absorption Parameters for 25 selected Bioactive Compounds

Sr no	**Ligand**	**Water solubility**	**$CaCO_2$ permeability**	**Intestinal Absorption**	**Skin Permeability**	**P-glycoprotein Substrate**	**P-glycoprotein I Inhibitor**	**P-glycoprotein II Inhibitor**
1.	Epicatechin	-3.117	-0.283	68.829	-2.735	Yes	No	No
2.	Querctin	-2.925	-0.229	77.207	-2.735	Yes	No	No
3.	Catechin	-3.117	-0.283	68.829	-2.735	Yes	No	No
4.	Kaempferol	-3.04	0.032	74.29	-2.735	Yes	No	No
5.	P-Coumaric acid	-2.378	1.21	93.494	-2.715	No	No	No
6.	Linoleic acid	-5.862	1.57	92.329	-2.723	No	No	No
7.	Palmitic acid	-5.562	1.558	92.004	-2.717	No	No	No
8.	Phytol	-7.535	1.399	90.643	-2.631	No	No	No
9.	Apigenin	-3.329	1.007	93.25	-2.735	Yes	No	No
10.	Luteolin	-3.094	0.096	81.13	-2.735	Yes	No	No
11.	Ferullic acid	-2.817	0.176	93.685	-2.72	No	No	No
12.	Gallic acid	-2.56	-0.081	43.374	-2.735	No	No	No
13.	Vanillic acid	-1.838	0.33	78.152	-2.726	No	No	No

Table 4.3 continued from previous page

Sr no	Ligand	Water solubility	$CaCO_2$ permeability	Intestinal Absorption	Skin Permeability	P-glycoprotein Substrate	P-glycoprotein I Inhibitor	P-glycoprotein II Inhibitor
14.	Caffeic acid	-2.33	0.634	69.407	-2.722	No	No	No
15.	Cucurbitacin E	-5.177	0.569	92.742	-3.457	Yes	Yes	Yes
16.	Cucurbitacin B	-5.046	0.588	89.52	-3.496	Yes	Yes	Yes
17.	Cucurbitacin L	-4.663	0.628	85.891	-3.577	Yes	Yes	Yes
18.	Cucurbitacin I	-4.683	0.649	85.113	85.113	Yes	Yes	Yes
19.	Cucurbitacin D	-4.548	0.682	81.891	-3.654	Yes	Yes	Yes
20.	Isochaftoside	-2.892	-1.18	45.385	45.385	Yes	No	No
21.	Chlorogenic acid	-2.449	-0.84	36.377	-2.735	Yes	No	No
22.	B-sitosterol	-6.773	1.201	94.464	-2.783	No	Yes	Yes
23.	Campestrol	-4.044	0.949	94.75	-2.96	Yes	No	No
24.	Alpha-tocopherol	-6.901	1.345	89.782	-2.683	No	Yes	Yes
25.	Stigmasterol	-6.682	1.213	94.97	-2.783	No	Yes	Yes

While analyzing the $CaCO_2$ permeability parameter, the model of intestinal wall permeability considered is > 0.90 regarded as high. Only the fatty acids (P-Coumaric acid, Linoleic acid, Palmitic acid), Phytols and Apigenin show high permeability. Most others are moderate to low.

Analysis of P-gp substrate/inhibitor shows that many flavonoids are P-gp substrates which may limit their oral bioavailability. The Cucurbitacins are major red flags here as they act as both substrates and inhibitors indicating high potential for Drug interaction and efflux.

4.2.1.3 Distribution

Once the compound enters the bloodstream, it is distributed in body's tissues and organs through process of circulation. The extent and rate of distribution are influenced by flow of blood, tissue permeability , binding affinity of plasma protein, and lipophilicity of compounds.

Only the small portion of drug i.e unbound fraction can pass membranes and reach extravascular tissues whereas drugs with high protein binding affinity may remain in plasma that may lead to their action to be prolonged. Even when plasma levels are similar variations in body compositions, age and disease states significantly alter the distribution rate.

Analysis of this section determines how the compound is distributed in the body including whether it reaches brain or not. Volume of distribution >0.45 L/kg suggests good distributions into tissues. Low VDss (< 0.15) suggests confinement to the bloodstream. Analysis shows compounds like Quercitin, luteolin and Isochaftoside have high VDss which suggests that they are best distributed to tissues. Many of acids such as P-Coumaric, Gallic, Vanillic acids have low VDss indicating they may stay in the systemic circulation.

While interpreting BBB permeability >-1.0 is high, < -1.0 is low considered respectively. Isochaftoside and Chlorogenic acid have very low CNS penetrations, while Sterols and Phytols have higher potential to enter the CNS.

For evaluating the Fraction Unbound (Fu) values , the proportion of drug not bound to plasma protein >0.1 is considered good, as only the unbound fractions is pharmacologically active. Analysis reveals that most compounds have a good Fu >0.1. A significant exception is Phytol and Sterol (B-sitosterol etc.)with Fu=0, meaning that they are highly protein-bound and may have low effective concentrations.

TABLE 4.4: Distribution Parameters for 25 Selected Bioactive Compounds

Sr no	**Ligand**	**VDss (human)**	**Fraction unbound (human)**	**BBB permeability**	**CNS permeability**
1.	Epicatechin	1.027	0.235	-1.054	-3.298
2.	Quercetin	1.559	0.206	-1.098	-3.065
3.	Catechin	1.027	0.235	-1.054	-3.298
4.	Kaempferol	1.274	0.178	-0.939	-2.228
5.	P-Coumaric acid	-1.151	0.428	-0.225	-2.418
6.	Linoleic acid	-0.587	0.054	-0.142	-1.6
7.	Palmitic acid	-0.543	0.101	-0.111	-1.816
8.	Phytol	0.385	0	0.793	-1.527
9.	Apigenin	0.822	0.147	-0.734	-2.061
10.	Luteolin	1.153	0.168	-0.907	-2.251
11.	Ferullic acid	-1.367	0.343	-0.239	-2.612
12.	Gallic acid	-1.855	0.617	-1.102	-3.74
13.	Vanillic acid	-1.739	0.518	-0.38	-2.628
14.	Caffeic acid	-1.098	0.529	-0.647	-2.608
15.	Cucurbitacin E	-0.322	0.1	-1.098	-3.013
16.	Cucurbitacin B	-0.348	0.124	-1.003	-3.029
17.	Cucurbitacin L	-0.374	0.205	-0.976	-3.443
18.	Cucurbitacin I	-0.387	0.21	-0.915	-3.43
19.	Cucurbitacin D	-0.408	0.233	-0.82	-3.447
20.	Isochaftoside	1.303	0.145	-1.671	-4.84
21.	Chlorogenic acid	0.581	0.658	-1.407	-3.856
22.	B-sitosterol	0.193	0	0.781	-1.705
23.	Campestrol	-0.226	0.11	-0.585	-2.332
24.	Alpha-tocopherol	0.709	0	0.876	-1.669
25.	Stigmasterol	0.178	0	0.771	-1.652

4.2.1.4 Metabolism

Metabolism means the biochemical transformation of compound in the body which is primarily carried out by the enzymes present in liver. After passing through this process drugs are converted into metabolites that may be active, inactive or sometimes toxic. This include two major phases functionalization (phase I) which often involves cytochrome P450 enzymes and conjugation (phase II) in which compounds are made more water soluble for easier excretion. Metabolic rates are significantly influenced by the factors such as genetics, age, disease and co-administered drugs. Infact recent research shows that interindividual variability in CYP450 enzyme expression remains a important determinant in safety and efficacy of drug [220].

Analysis of metabolism parameters is interpreted based on "Yes" for being a substrate means the body will likely metabolize and clear the compound quickly and "Yes" for being an inhibitor is a major red flag for drug-drug ineteraction as it can slow down the metabolism of other co-administered drugs. Evaluatiom reveals that Epicatechin, Catechin and simple acid (P-Coumaric, Ferullic, Gallic, Vanillic, Caffeic) show no CYP interaction, means they form cleanest profiles and Cucurbitacins are all CYP3A4 substrates meaning they will be rapidly metabolized. Moreover Linoleic acid, Phytol, Alpha-tocopherol is CYP3A4 inhibtors posing a high risk for drug interactions. Quercitin, Kaempferol, Apigenin, campestrol and luteolin show moderate concerns.

TABLE 4.5: Metabolism Parameters for 25 Selected Bioactive Compounds

Sr.	**Ligands**	**Substrate**		**Inhibitor**				
		CYP2D6	**CYP3A4**	**CYP1A2**	**CYP2C19**	**CYP2C9**	**CYP2D6**	**CYP3A4**
1.	Epicatechin	NO	NO	NO	NO	NO	NO	NO
2.	Quercetin	No	No	Yes	No	No	No	No
3.	Catechin	NO	NO	NO	NO	NO	NO	NO
4.	Kaempferol	No	No	Yes	No	No	No	No
5.	P-Coumaric acid	No	No	No	No	No	No	No
6.	Linoleic acid	No	Yes	Yes	No	No	No	No
7.	Palmitic acid	No	Yes	No	No	No	No	No
8.	Phytol	No	Yes	Yes	No	No	No	No

Table 4.5 continued from previous page

Sr.	Ligands	Substrate		Inhibitor				
		CYP2D6	**CYP3A4**	**CYP1A2**	**CYP2C19**	**CYP2C9**	**CYP2D6**	**CYP3A4**
9.	Apigenin	No	No	Yes	Yes	No	No	No
10.	Luteolin	No	No	Yes	No	Yes	No	No
11.	Ferullic acid	No	No	No	No	No	No	No
12.	Gallic acid	No	No	No	No	No	No	No
13.	Vanillic acid	No	No	No	No	No	No	No
14.	Caffeic acid	No	No	No	No	No	No	No
15.	Cucurbitacin E	No	Yes	No	No	No	No	No
16.	Cucurbitacin B	No	Yes	No	No	No	No	No
17.	Cucurbitacin L	No	Yes	No	No	No	No	No
18.	Cucurbitacin I	No	Yes	No	No	No	No	No
19.	Cucurbitacin D	No	Yes	No	No	No	No	No
20.	Isochaftoside	No	No	No	No	No	No	No
21.	Chlorogenic acid	No	No	No	No	No	No	No
22.	B-sitosterol	No	Yes	No	No	No	No	No
23.	Campestrol	No	Yes	Yes	No	No	No	No
24.	Alpha-tocopherol	No	Yes	No	Yes	No	No	No
25.	Stigmasterol	No	Yes	No	No	No	No	No

4.2.1.5 Excretion

Excretion is the crtical and final step of pharmacokinetics, in which drugs and their metabolites are eliminated from the body. The kidneys are major organs involved in elimination of these compounds through urine, while other pathways include bile, feces, sweatt and exhaled air. Drug solubility, molecular size and renal or hepatic processes affect the efficiency of excretion. Poorly metabolized compounds are often excreted unchanged, however compounds undergoing extensive metabolism are eliminated as more hydrophilic conjugates. Also impaired renal clearance can significantly affect the drug exposure and even toxicity risks which make excretion studies more essential in early drug discovery [221].

A higher value indicates the faster clearance. Fatty acids (Linoleic, Palmitic) and phytols have very high clearance. The Cucurbitacins and Isochaftoside have very

low clearance, which means it could lead to accumulation and potential toxicity with repeated dosing.

TABLE 4.6: Excretion Parameters for 25 Selected Bioactive Compounds

Sr no.	**Ligand**	**Total Clearance**	**Renal OCT**
1.	EPICATECHIN	0.183	No
2.	Quercetin	0.407	No
3.	Catechin	0.183	No
4.	Kaempferol	0.477	No
5.	P-Coumaric acid	0.662	No
6.	Linoleic acid	1.936 highest among all	No
7.	Palmitic acid	1.763	No
8.	Phytol	1.686	No
9.	Apigenin	0.566	No
10.	Luteolin	0.495	No
11.	Ferullic acid	0.632	No
12.	Gallic acid	0.518	No
13.	Vanillic acid	0.628	No
14.	Caffeic acid	0.508	No
15.	Cucurbitacin E	0.096	No
16.	Cucurbitacin B	0.137	No
17.	Cucurbitacin L	0.173	No
18.	Cucurbitacin I	0.188	No
19.	Cucurbitacin D	0.276	No
20.	Isochaftoside	-0.11	No
21.	Chlorogenic acid	0.307	No
22.	B-sitosterol	0.628	No
23.	Campestrol	0.609	No
24.	Alpha-tocopherol	0.794	No
25.	Stigmasterol	0.618	No

4.2.1.6 Toxicity

In addition to pharmacokinetic properties, evaluation of toxicity is also an essential part of early drug discovery as it helps to identify compounds which may cause dangerous effects. Actually Toxicity refers to the potential of compound to produce

undesireable results such as liver damage , genetic mutations, cardiac problems, and some other systemic effects.

Computational tools help researchers to predict these parameters very efficiently by determining molecular structures and comparing them with already existing toxic datasets.

In this process factors such as AMES mutagenicity, hepatotoxicity, maximum tolerated dose, hERG inhibition are considered more precisely to evaluate the safety profile of selected ligands this predictive modeling helps us to reduce the late stage failures risks and ensures that compounds with acceptable safety margin are short-listed only. In silico toxicity prediction techniques has become indispensable part of rational drug design which provides a quicker and cost effective alternative to traditional lab testings [222].

AMES toxicity evaluation is done by predicting mutagenicity (DNA damage). A "Yes" is a critical failure. Analysis reveals that only Cucurbitacin D is predicted as mutagenic, so this compound is eliminated from further consideration.

For Max tolerated dose $>$ 0.477 log mg/kg/day is considered high and potentially toxic. Analysis reveals that many compounds are in the safe range. However P-Coumaric acid, Ferullic acid, Caffeic acid have very high values, which suggests low risk of acute toxicity. The negative values for Cucurbitacin family and fatty acids are unusual but generally give indication of lower dose limits.

hERG inhibition predicts potential for cardiotoxicity, "Yes" is ofcourse a failure. Analysis reveals that Phytols, B-sitosterols, Alpha-tocopherols and Stigmasterols are predicted hERG II inhibitors. This is a serious red flag for these compounds.

Analysis of hepatotoxicity and skin sensitization reveals that Linoleic acid is flagged for both hepatotoxicity and skin sensitization. Campestrol is flagged for hepatotoxicity.

Environmental toxicity models for *T.Pyriforms* & Minnow Toxicity are *T.Pyriforms* $>$ -0.5 and Minnow Toxicity $>$-0.3 which indicates toxicity. Analysis reveals that Phytol , alpha-tocopherol show high toxicity in these models.

TABLE 4.7: Toxicity Parameters for 25 Selected Bioactive Compounds

Sr no	Ligands	AMES toxicity	Max. tolerated dose (human)	hERG I Inh	hERG II Inh	Oral Rat Acute Toxicity (LD50)	Oral Rat Chronic Toxicity (LOAEL)	Hepato toxicity	Skin Sensitisation	*T. Pyriformis* toxicity	Minnow toxicity
1.	Epicatechin	NO	0.438	NO	NO	2.428	2.5	NO	NO	0.347	3.585
2.	Quercetin	No	0.499	No	No	2.471	2.612	No	No	0.288	3.721
3.	Catechin	No	0.438	No	No	2.428	2.5	No	No	0.347	3.585
4.	Kaempferol	No	0.531	No	No	2.449	2.505	No	No	0.312	2.885n
5.	PCoumaric acid	No	1.111	No	No	2.155	2.534	No	No	0.319	1.607
6.	Linoleic acid	No	0.827	No	No	1.429	3.187	Yes	Yes	0.701	-1.31
7.	Palmitic acid	No	0.708	No	No	1.44	3.181	No	Yes	0.84	1.083
8.	Phytol	No	0.301	No	Yes	1.848	1.232	No	Yes	1.714	1.137
9.	Apigenin	No	0.328	No	No	2.45	2.298	No	No	0.38	2.432
10.	Luteolin	No	0.499	No	No	2.455	2.409	No	No	0.326	3.169
11.	Ferullic acid	No	1.082	No	No	2.282	2.065	No	No	0.271	1.825
12.	Gallic acid	No	0.7	No	No	2.218	3.06	No	No	0.285	3.188
13.	Vanillic acid	No	0.719	No	No	2.454	2.032	No	No	0.265	1.926

Table 4.7 continued from previous page

Sr no	Ligands	AMES toxicity	Max. tolerated dose (human)	hERG I Inh	hERG II Inh	Oral Rat Acute Toxicity (LD50)	Oral Rat Chronic Toxicity (LOAEL)	Hepato toxicity	Skin Sensitisation	*T. Pyriformis* toxicity	Minnow toxicity
14.	Caffeic acid	No	1.145	No	No	2.383	2.092	No	No	0.293	2.246
15.	Cucurbitacin E	No	-0.948	No	No	2.471	1.664	No	No	0.289	1.58
16.	Cucurbitacin B	No	-0.928	No	No	2.381	1.709	No	No	0.288	1.731
17.	Cucurbitacin L	No	-0.701	No	No	2.644	1.463	No	No	0.287	1.978
18.	Cucurbitacin I	No	-0.725	No	No	2.655	1.484	No	No	0.287	2.105
19.	Cucurbitacin D	Yes	-0.71	No	No	2.555	1.529	No	No	0.287	2.256
20.	Isochaftoside	No	0.464	No	Yes	2.495	4.817	No	No	0.285	9.333
21.	Chlorogenic acid	No	-0.134	No	No	1.973	2.982	No	No	0.285	5.741
22.	B-sitosterol	No	-0.621	No	Yes	2.552	0.855	No	No	0.43	-1.802
23.	Campestrol	No	-0.047	No	No	2.447	2.051	yes	No	0.715	0.435
24.	Alpha-tocopherol	No	0.775	No	Yes	2.072	1.987	No	No	1.017	-3.324
25.	Stigmasterol	No	-0.664	No	Yes	2.54	0.872	No	No	0.433	-1.675

4.3 Molecular Docking

Three-dimensional structures of ligands and proteins are used for the purpose of molecular docking. A very high throughput software PyRx is used for docking of receptors and ligands. PyRX can be used to dock multiple ligands at the same time and gives us the best pose of ligands that bind to target site of proteins. Results of molecular docking can be analyzed by determination of ligand position and quantitative analysis can be done by monitoring scoring alogrithms. Binding energy is directly proportional to the successful docking of protein and ligand.

While using the PyRx ,RMSD value is used to find the accurate perception of docking results by comparing of ligand docked pose with a reference pose (experimentally determined). The lower the value of RMSD is the more accurate docking result is. A reliable docking result often shows a RMSD value below 2A and zero is considered as ideal value [224].

TABLE 4.8: Molecular Docking of Selected 25 Bioactive Compounds

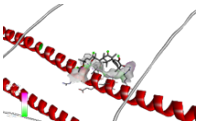
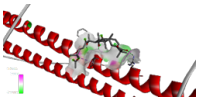
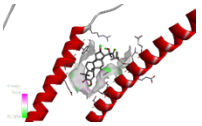
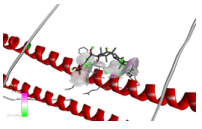
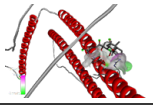
Sr no	**Ligand Name**	**Binding Affinity**	**RMS Value**	**3D Structure**
1.	Cucurbitacin E.	-5.5	0	
2.	Cucurbitacin B.	-6.7	0	
3.	Cucurbitacin L	-6	0	
4.	Cucurbitacin I	-6.4	0	
5.	Cucurbitacin D.	-5.7	0	

Table 4.8 continued from previous page

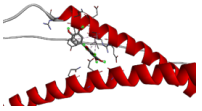
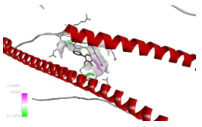
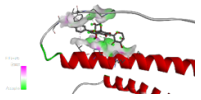
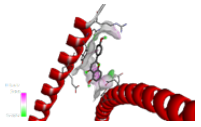
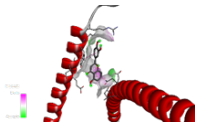
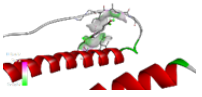
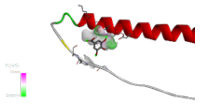
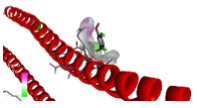
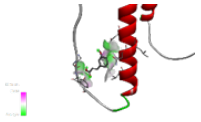
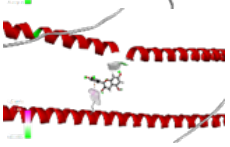
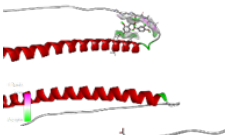
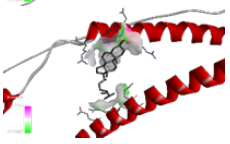
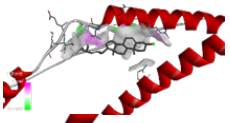
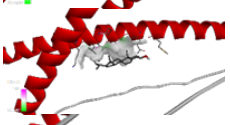
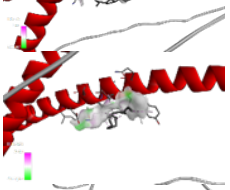
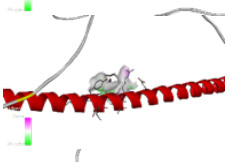
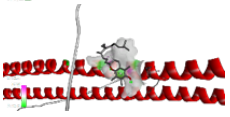
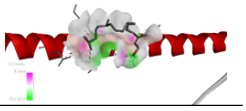
Sr no	Ligand Name	Binding Affinity	RMS Value	3D Structure
6.	Quercetin.	-5.8	0	
7.	Kaempferol.	-5.7	0	
8.	Isoschaftoside.	-6	0	
9.	Apigenin.	-5.5	0	
10.	Luteolin.	-6.1	0	
11.	Ferulic acid.	-4.3	0	
12.	Gallic acid.	-3.8	0	
13.	Vanillic acid.	-3.9	0	
14.	Caffeic acid.	-4.4	0	
15.	Chlorogenic acid.	-4.7	0	

Table 4.8 continued from previous page

Sr no	Ligand Name	Binding Affinity	RMS Value	3D Structure
16.	P-Coumaric acid.	-4.9	0	
17.	Catechin.	-5.7	0	
18.	Epicatechin.	-5.9	0	
19.	B-sitosterol.	-6.7	0	
20.	Stigmasterol.	-6.4	0	
21.	Campesterol.	-7.3	0	
22.	Linoleic acid.	-3.9	0	
23.	Palmitic acid.	-2.8	0	
24.	Alpha-tocopherol	-5.7	0	
25.	Phytol	-3.8	0	

4.3.1 Analysis and Visualization of Protein Ligand Interactions

The most beneficial ligand configuration was selected based on the greatest binding capacity values observed in Discovery Studio after molecular docking using PyRx. The constructed protein structure and the chosen posture were then loaded into

Discovery Studio for analysis of interaction after being saved in PDB format. Hydrogen bonding, hydrophobic interaction, and electrostatic forces were identified to analyze the protein–ligand interactions.

The bond lengths involved in hydrogen bonding were also noted. This interaction study helped to optimize the ligand's structure and revealed important information regarding the ligand's binding behaviors and potential mode of action.

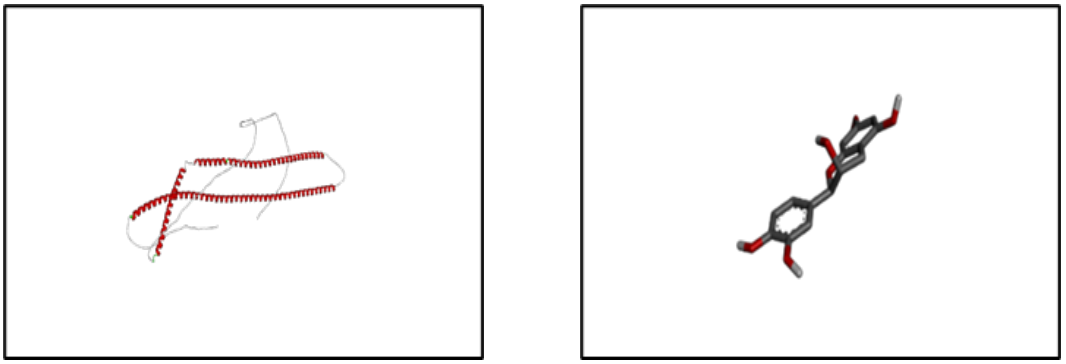
For analysis of molecular docking results, 2D structure is analyzed which shows different types of bondings that include hydrogen bond, hydrophobic bond and electrostatic bond between protein and ligands.

Among selected ligands, Campestrol shows highest binding energy of -7.3 kcal/mol and has total 10 bonds which includes 2 Hydrogen bonds, 7 hydrophobic bonds and 1 electrostatic bond.

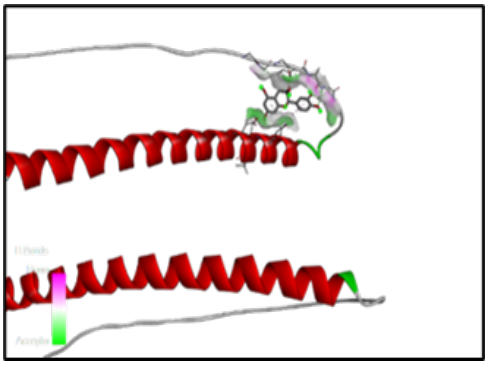
Cucurbitacin B & I, Luteolin, B-sitosterol, and Stigmasterol shows high binding energies in range -6.1 to -6.7 kcal/mol in which cucurbitacin B form total 4 hydrogen bonds, cucurbitacin I forms 3 hydrogen bonds and 2 hydrophobic bonds, B-sitosterol form 1 hydrogen bond and 3 hydrophobic bonds, and stigmasterol form 5 hydrophobic bonds.

Whereas Cucurbitacin E, Cucurbitacin D. Quercetin., Kaempferol, Apigenin., Catechin, Epicatechin and Alpha-tocopherol shows moderate binding energies in range -5.0 to -6.0kcal/mol in which Cucurbitacin E forms 4 hydrogen bonds and 1 hydrophobic bond, Cucurbitacin D forms 2 hydrophobic bonds and 2 hydrogen bonds, Quercitin forms 4 Hyrdrophobic bonds, Kaempferol forms 2 Hydrogen bonds and 3 hydrophobic bonds, Apigenin forms 1 Hydrogen bonds , 1 electrostatic bond and 4 Hydrophobic bonds, Epicatchin forms 2 Hydrogen bonds and 2 Hydrophobic bonds, Catechin forms 2 hydrogen bonds and 3 hydrophobic bonds.

Ferulic acid, Caffeic acid, Chlorogenic acid, P-Coumaric acid shows binding energies in range -4.0 to -4.9 kcal/mol whereas binding energies of Linoleic acid, phytol, Palmitic acid, Vanillic acid, Gallic acid, falls in lowest range of -2.8 kcal/mol to -3.9 kcal/mol.



(a) (b)



(c)

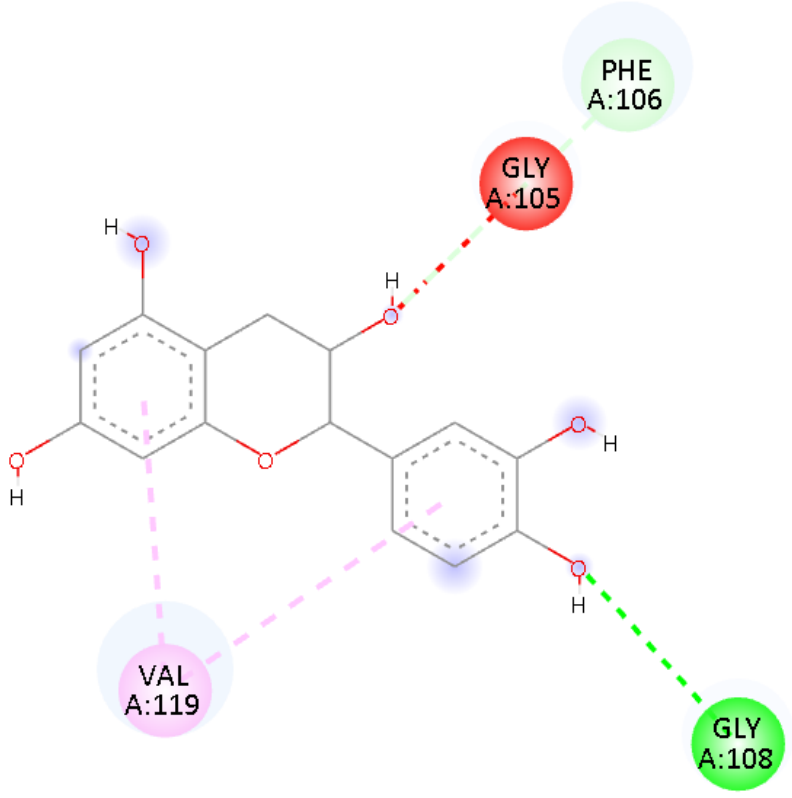


FIGURE 4.2: Analysis of molecular docking of KRT16 protein and Epicatechin (a) Structure of Human KRT16 protein (b)3D structure of Epicatechin (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

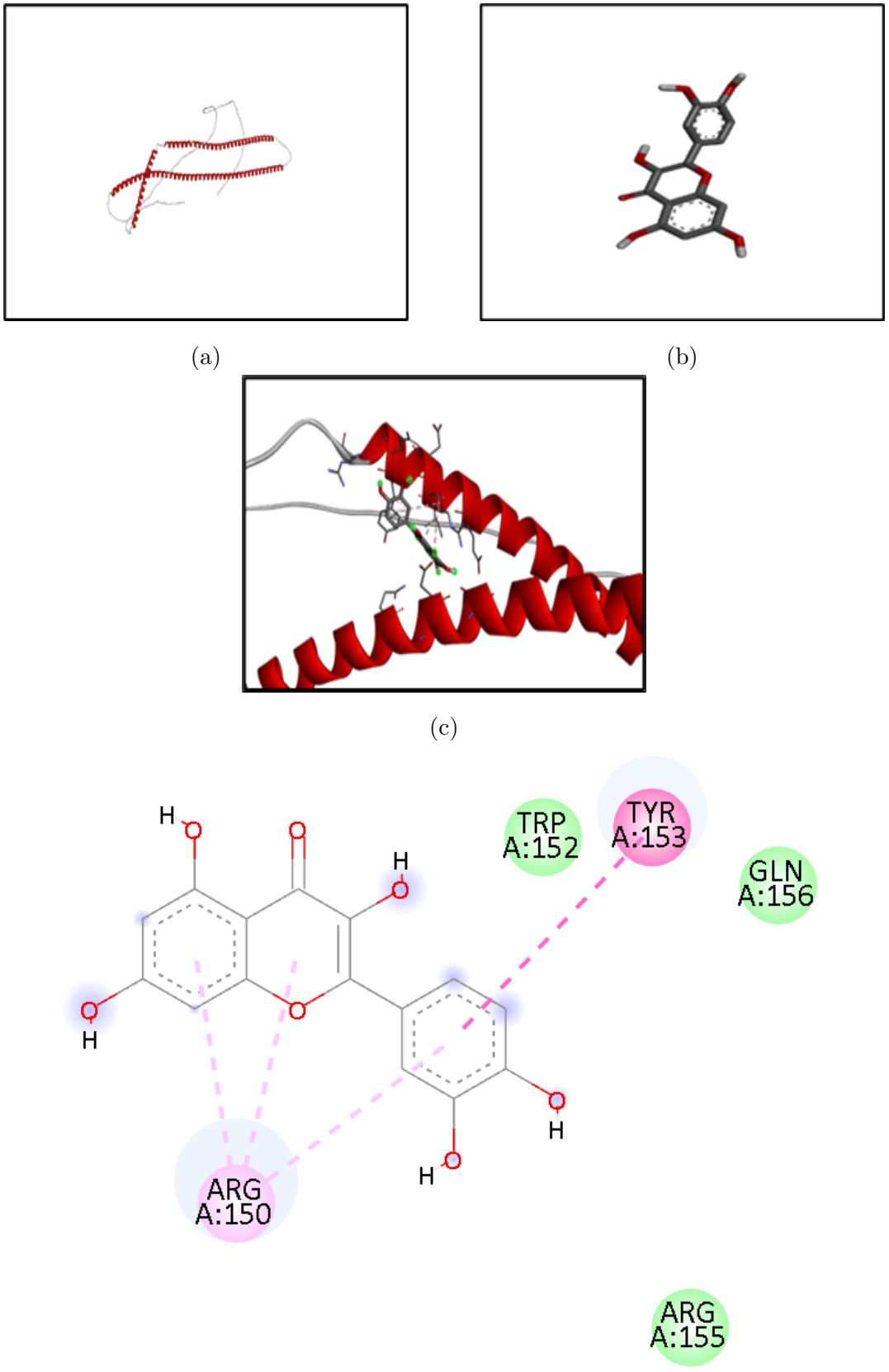
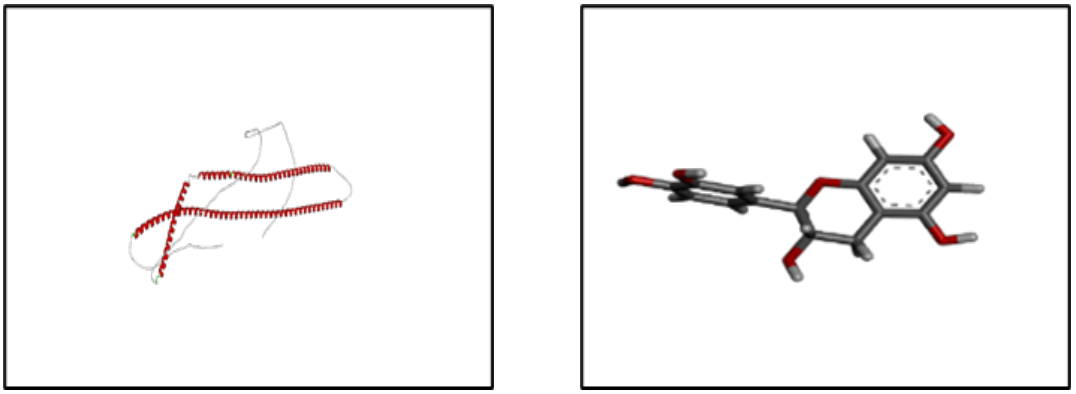
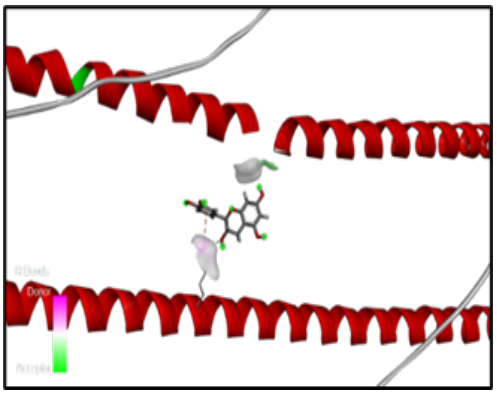


FIGURE 4.3: Analysis of molecular docking of KRT16 protein and Quercitin (a) Structure of Human KRT16 protein (b)3D structure of Quercitin (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.



(a) (b)



(c)

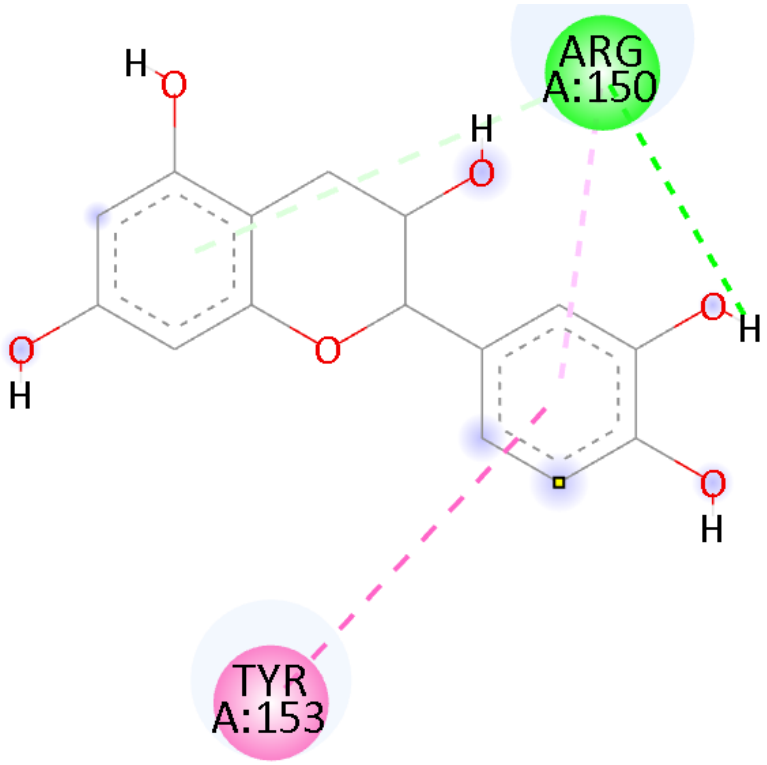


FIGURE 4.4: Analysis of molecular docking of KRT16 protein and Catechin (a) Structure of Human KRT16 protein (b)3D structure of catechin (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

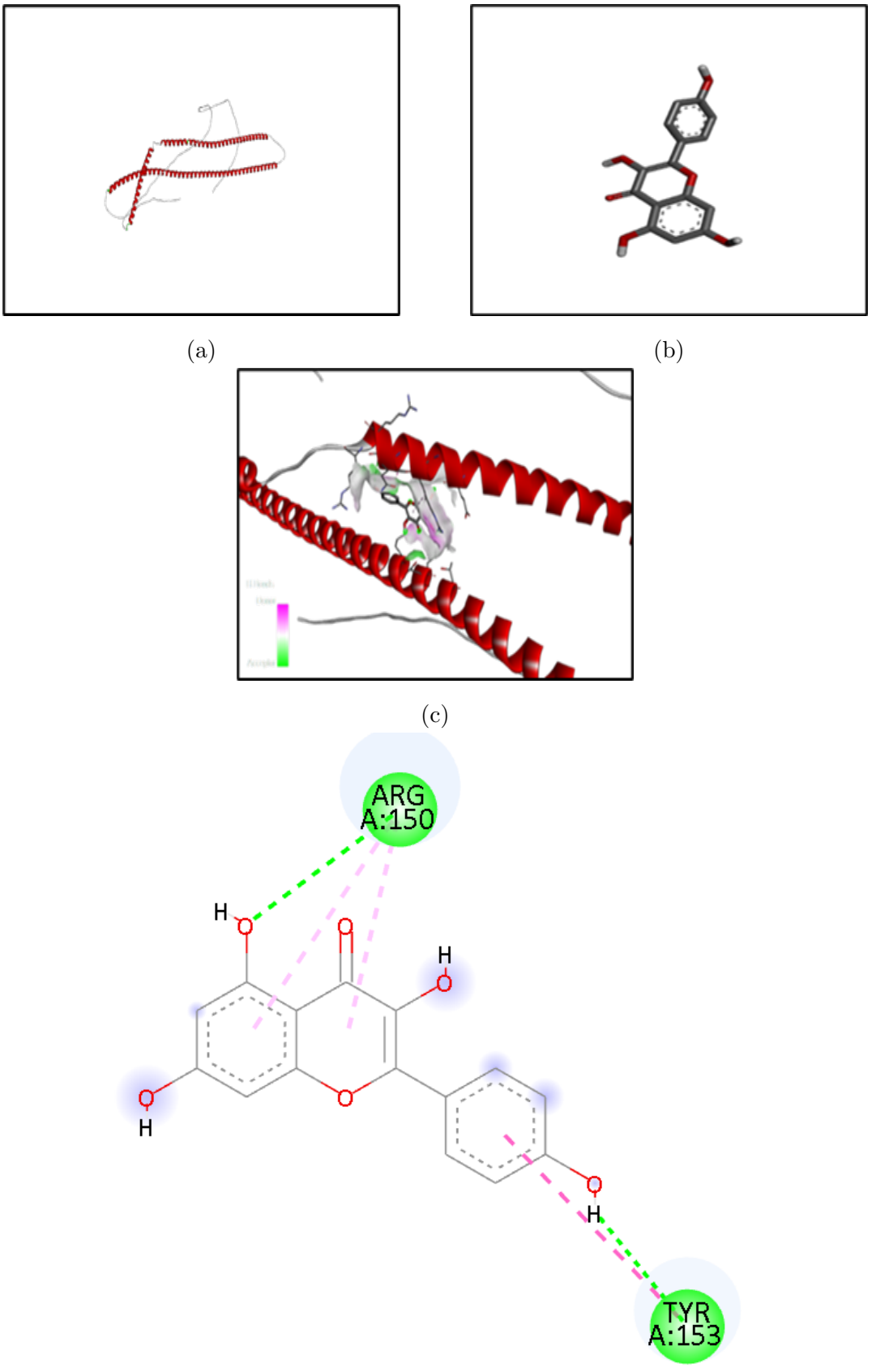


FIGURE 4.5: Analysis of molecular docking of KRT16 protein and Kaempferol (a) Structure of Human KRT16 protein (b)3D structure of Kaempferol (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

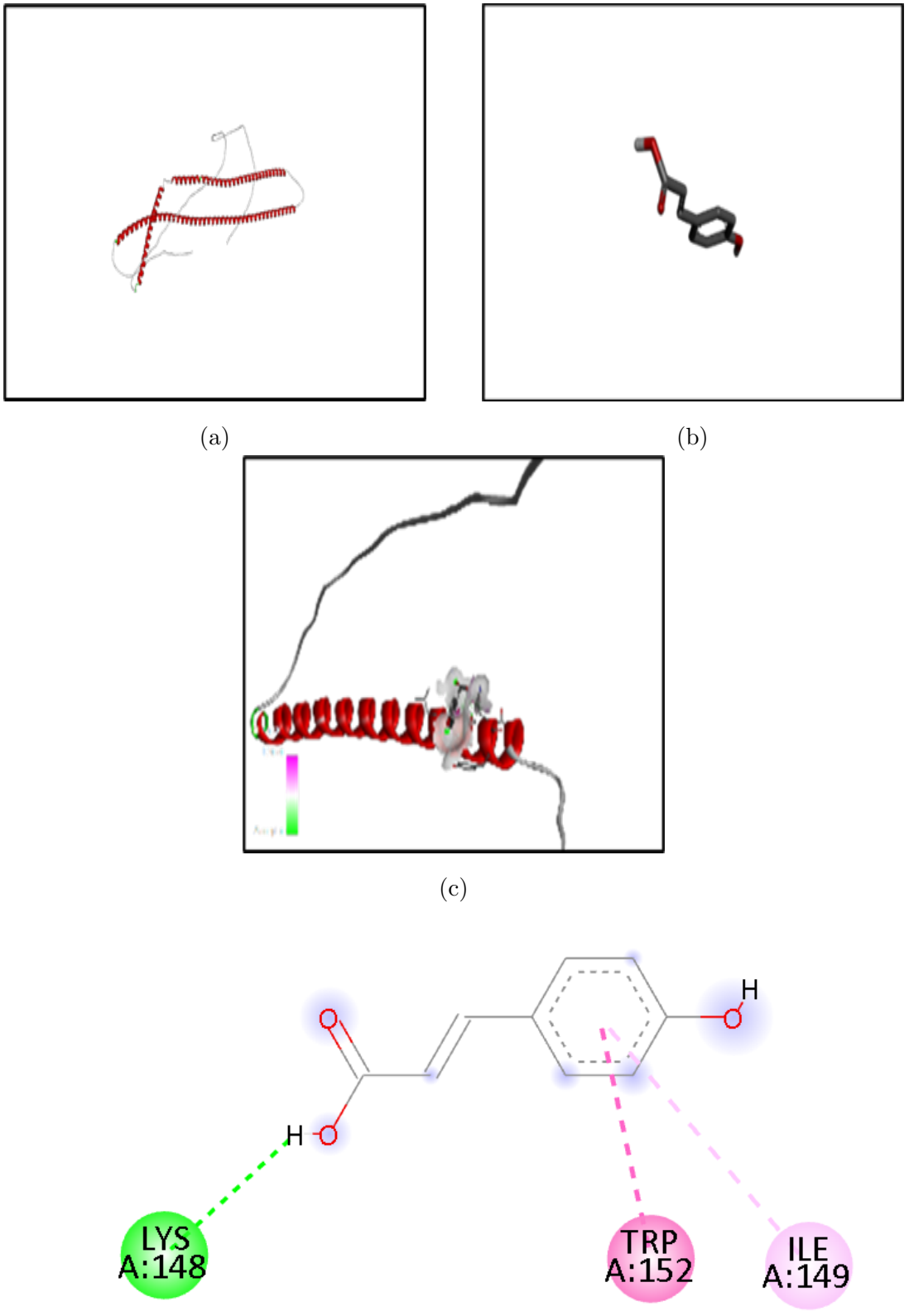


FIGURE 4.6: Analysis of molecular docking of KRT16 protein and P-coumaric acid (a) Structure of Human KRT16 protein (b)3D structure of P-coumaric acid (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

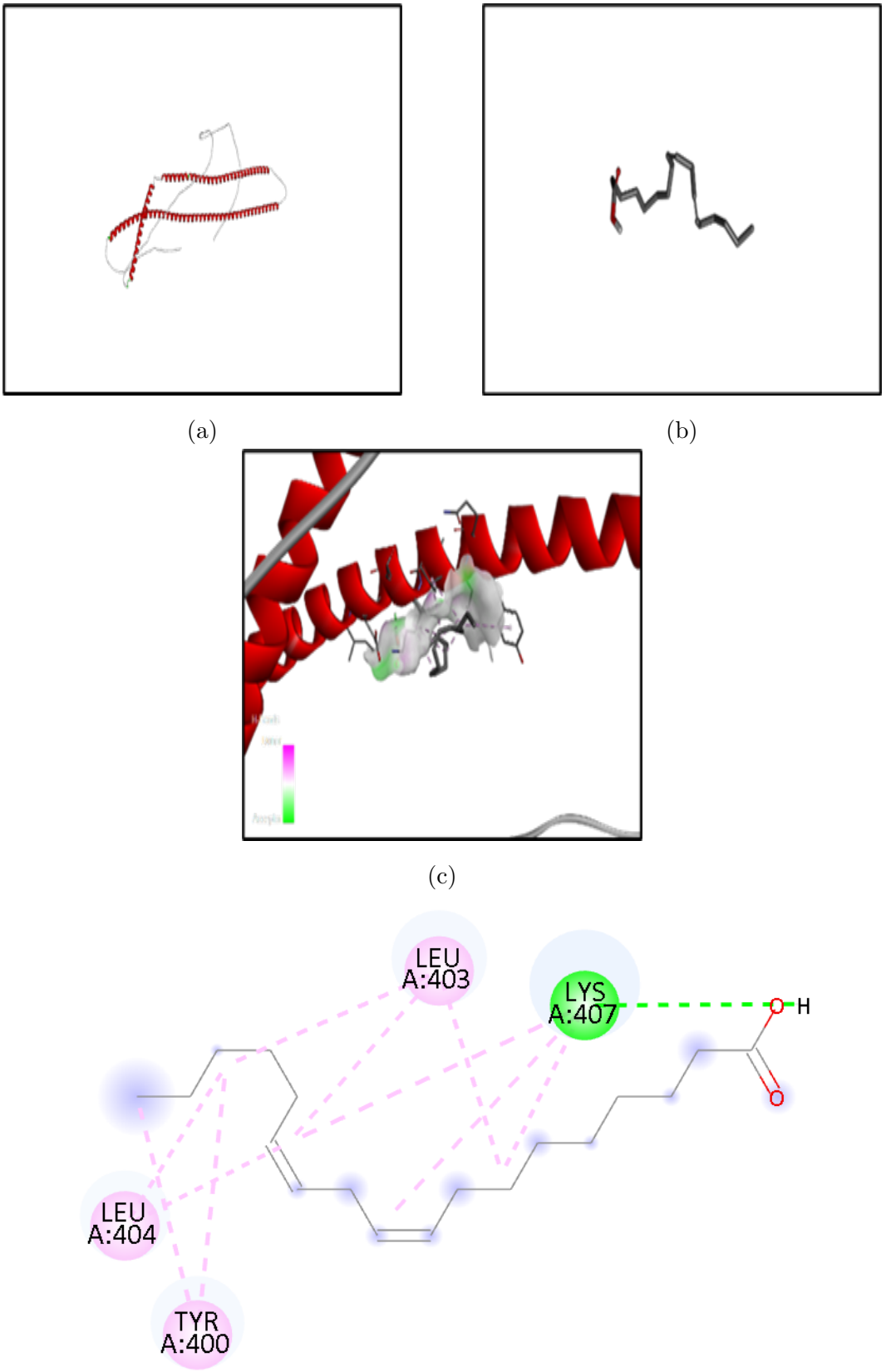


FIGURE 4.7: Analysis of molecular docking of KRT16 protein and Linoleic acid (a) Structure of Human KRT16 protein (b) 3D structure of Linoleic acid (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

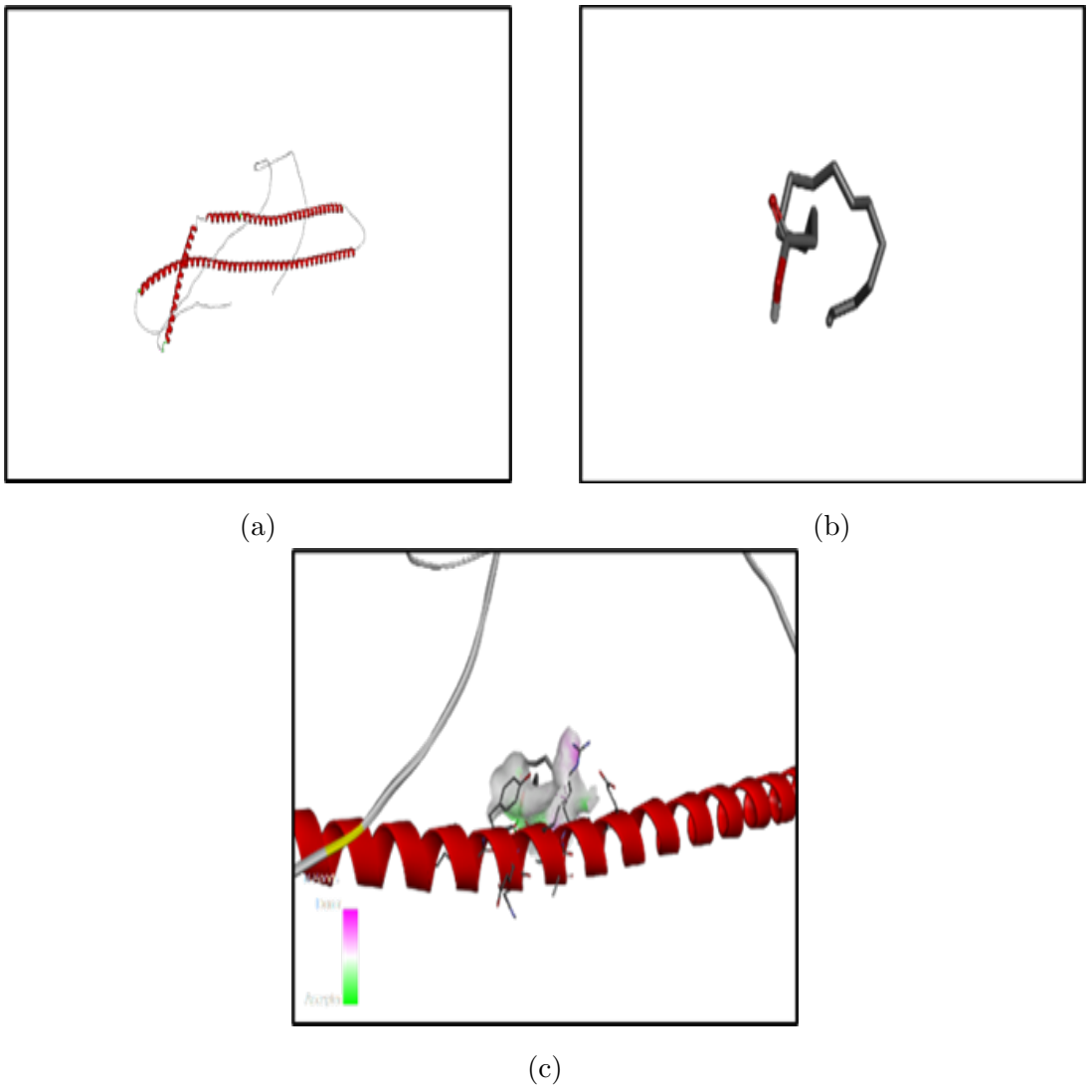


FIGURE 4.8: Analysis of molecular docking of KRT16 protein and Palmitic acid (a) Structure of Human KRT16 protein (b) 3D structure of Palmitic acid (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

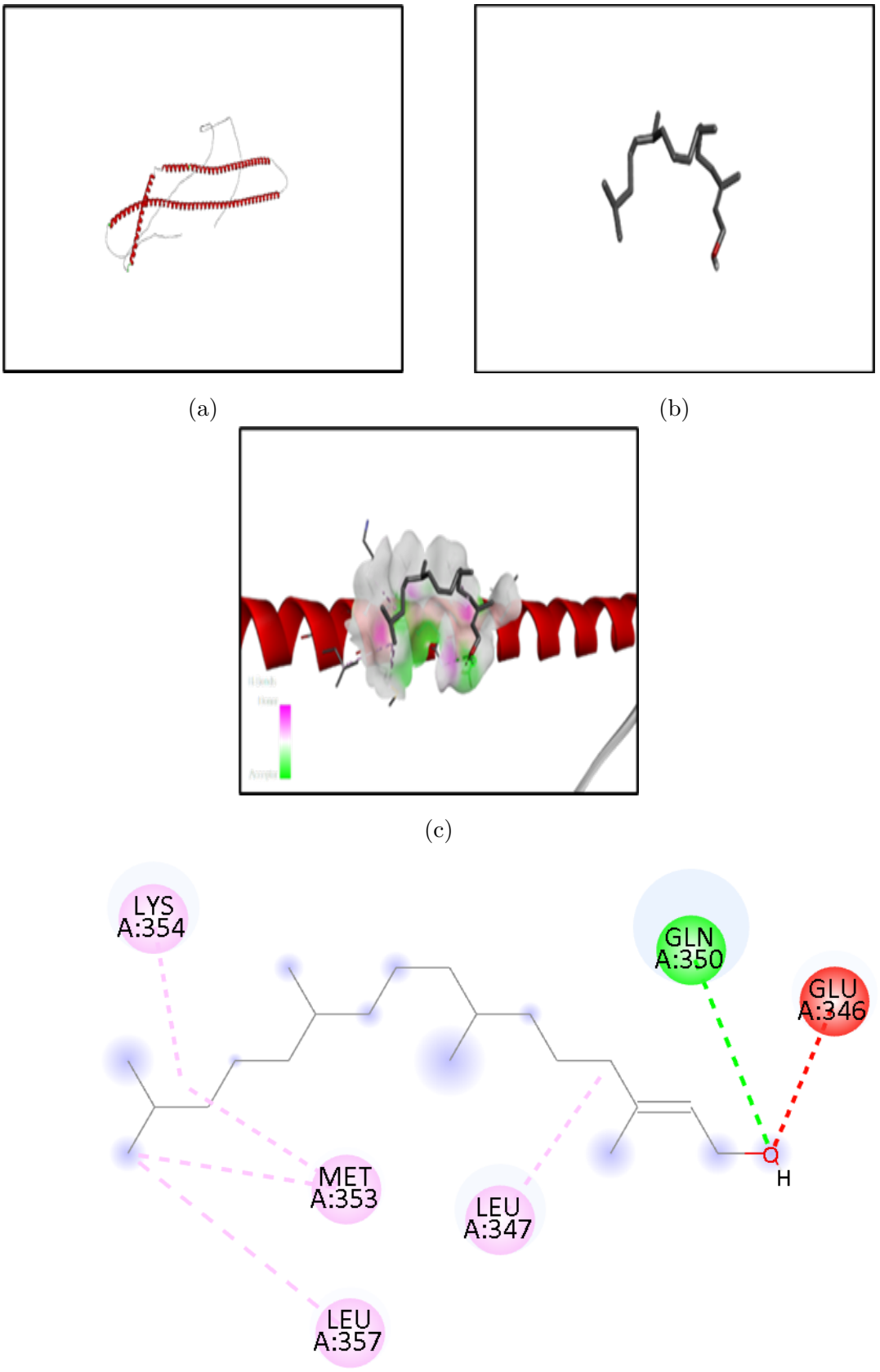


FIGURE 4.9: Analysis of molecular docking of KRT16 protein and Phytol (a) Structure of Human KRT16 protein (b) 3D structure of Phytol (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

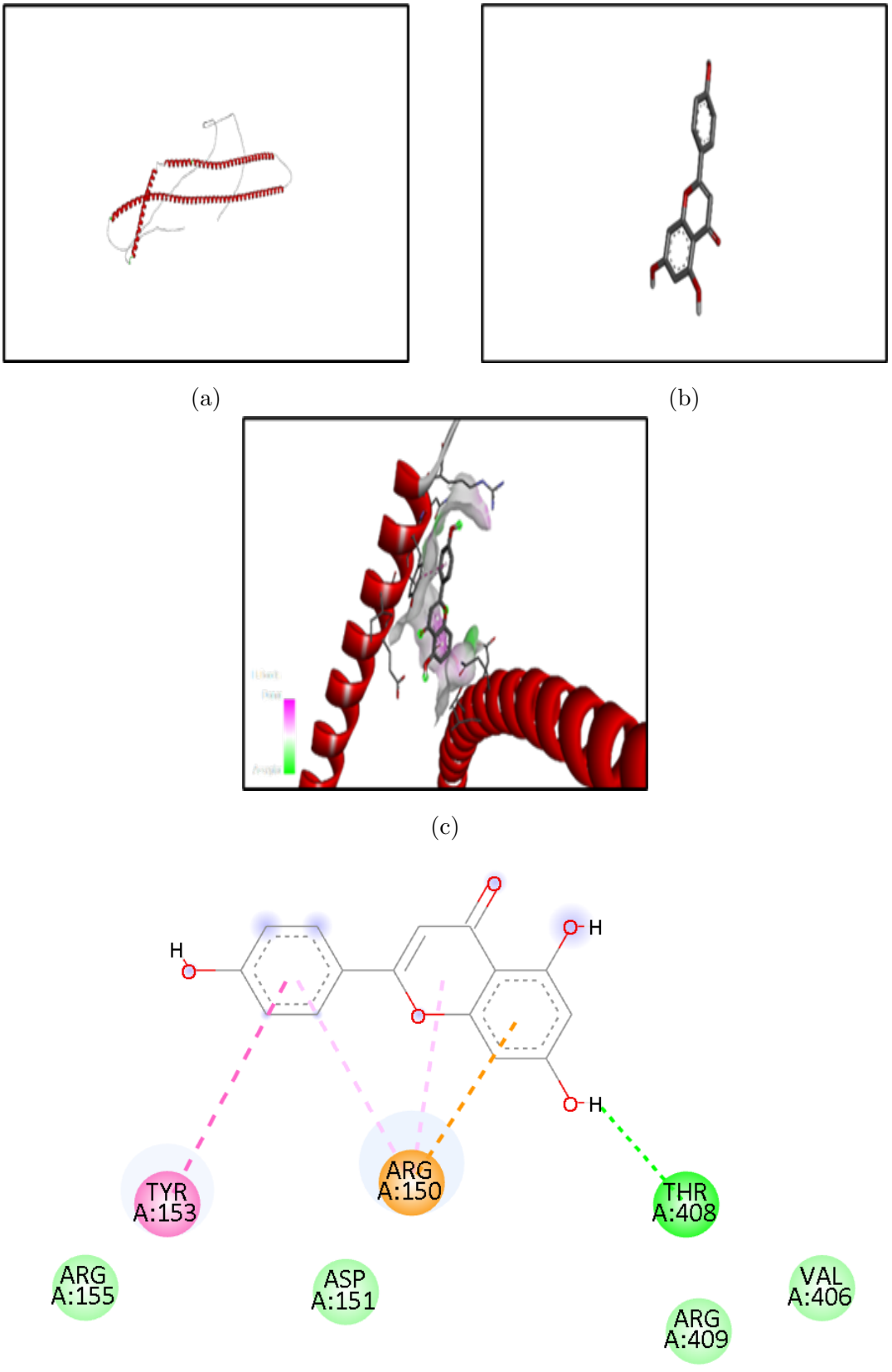


FIGURE 4.10: Analysis of molecular docking of KRT16 protein and Apigenin (a) Structure of Human KRT16 protein (b)3D structure of Apigenin (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

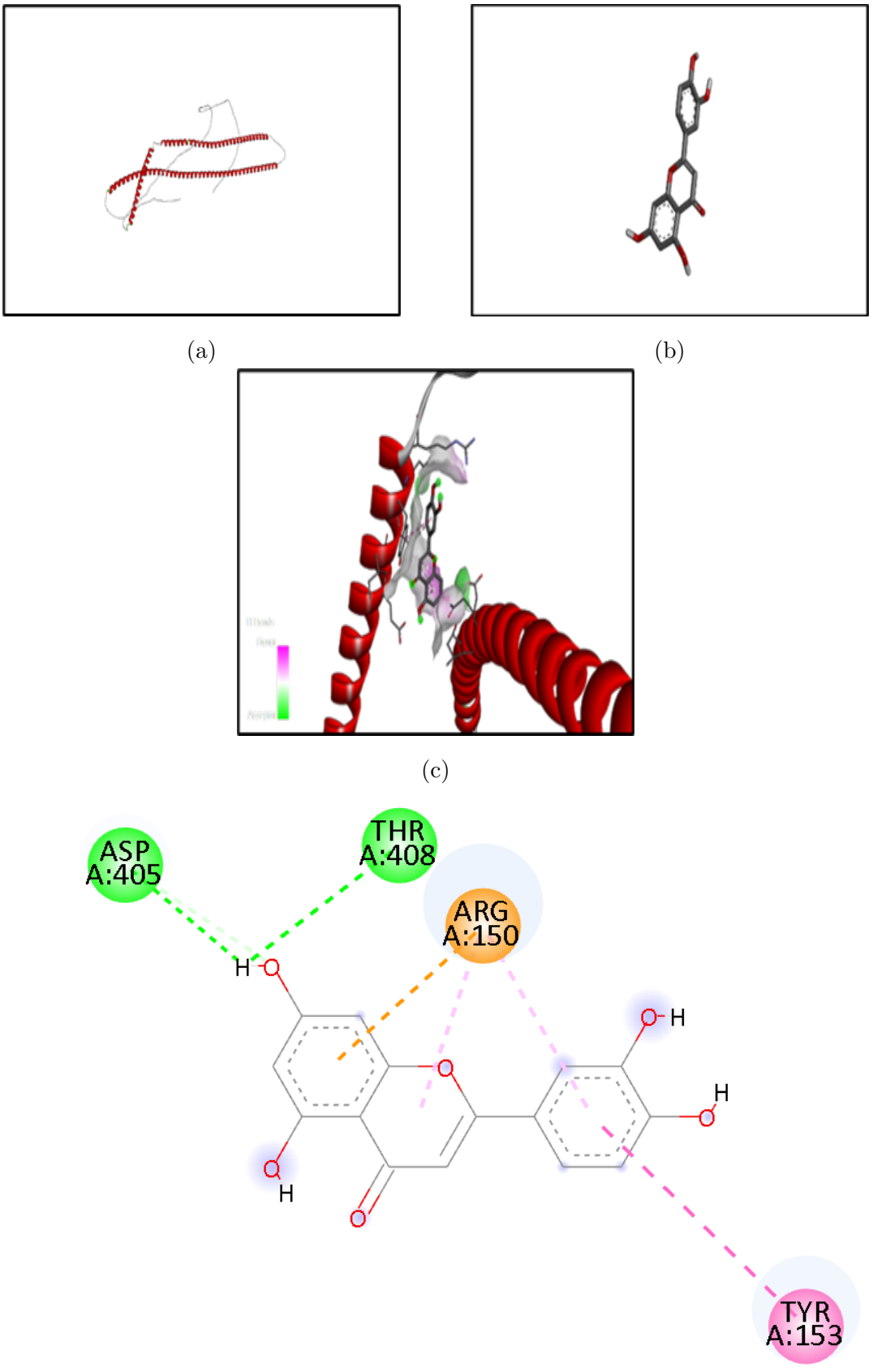


FIGURE 4.11: Analysis of molecular docking of KRT16 protein and Luteolin (a) Structure of Human KRT16 protein (b) 3D structure of Luteolin (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

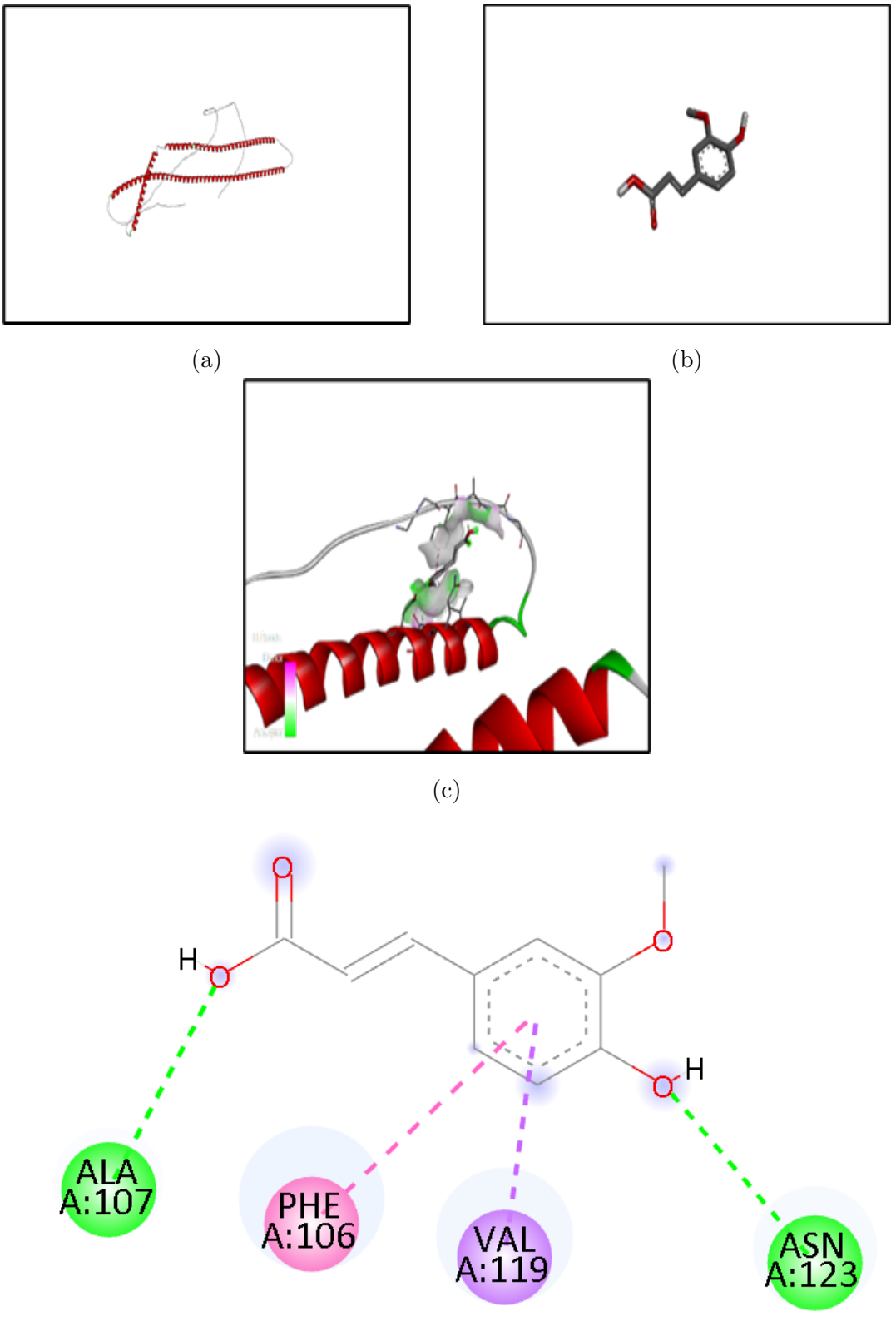


FIGURE 4.12: Analysis of molecular docking of KRT16 protein and Ferullic acid (a) Structure of Human KRT16 protein (b)3D structure of Ferullic acid (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

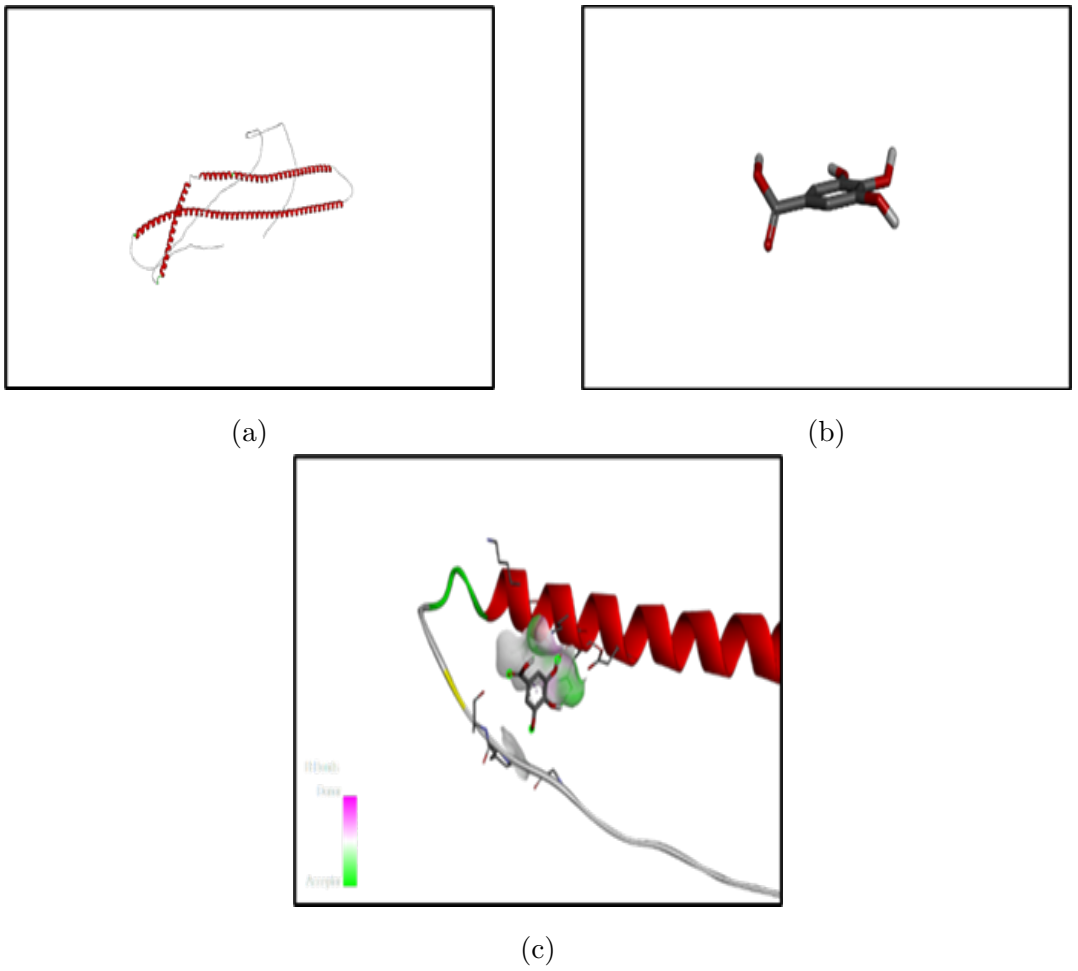


FIGURE 4.13: Analysis of molecular docking of KRT16 protein and Gallic acid (a) Structure of Human KRT16 protein (b)3D structure of Gallic acid (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

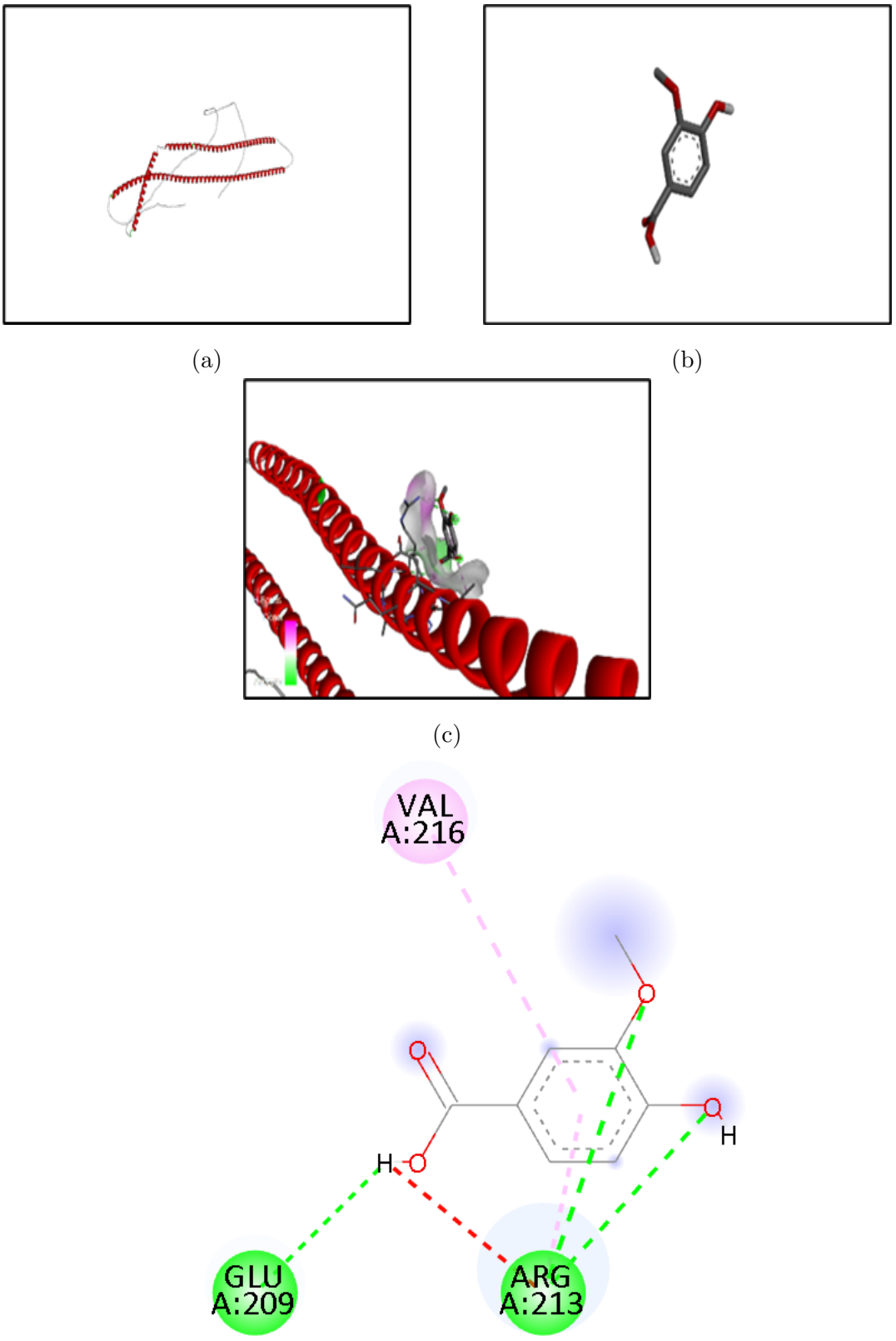


FIGURE 4.14: Analysis of molecular docking of KRT16 protein and Vanillic acid (a) Structure of Human KRT16 protein (b)3D structure of Vanillic acid (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

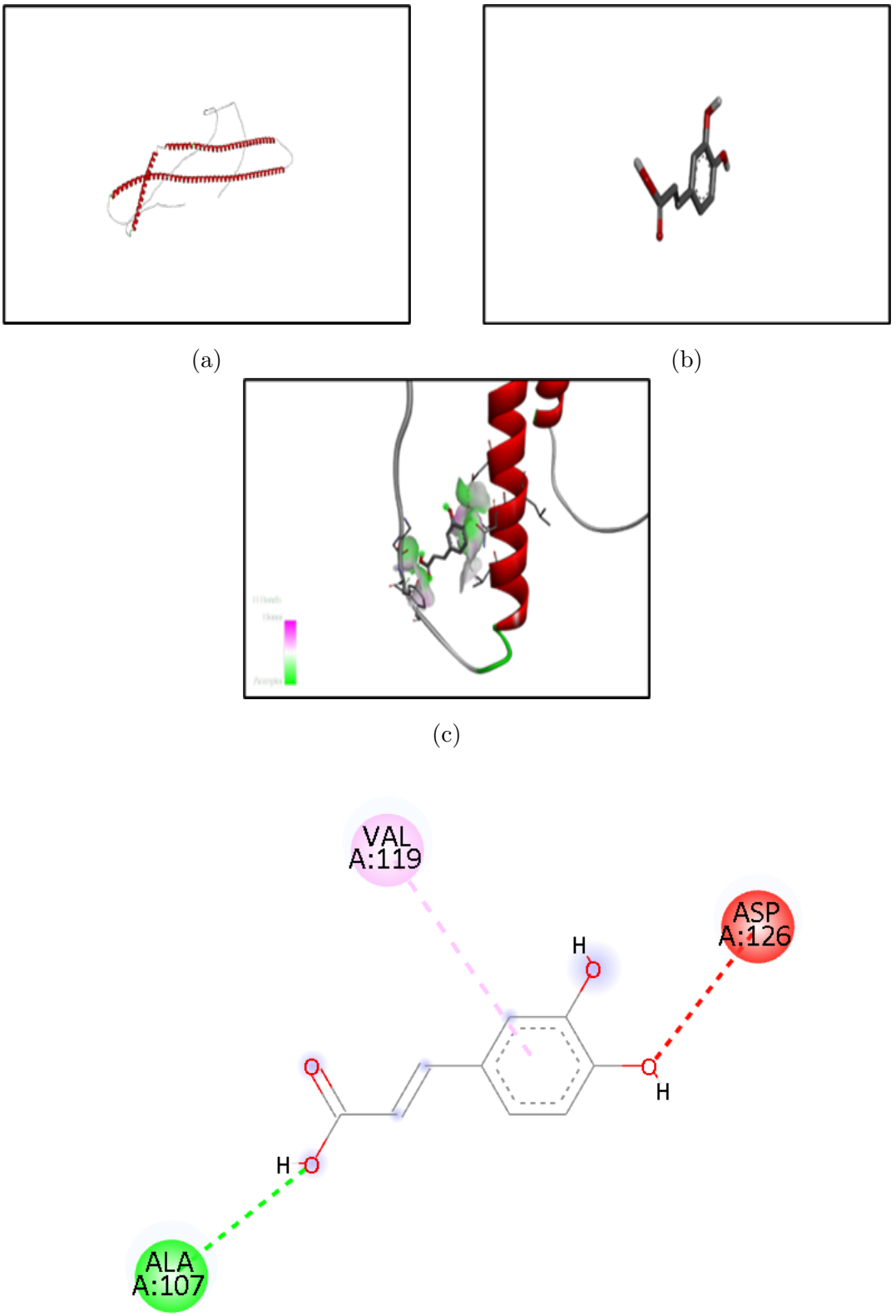
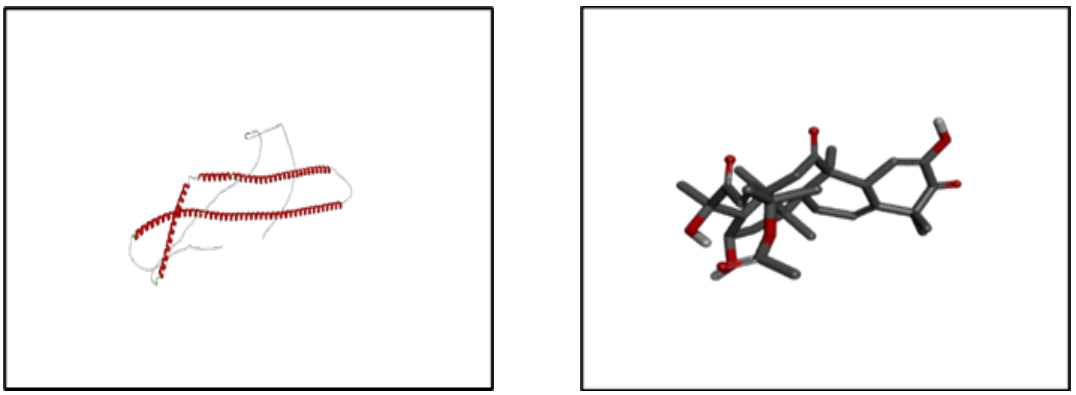
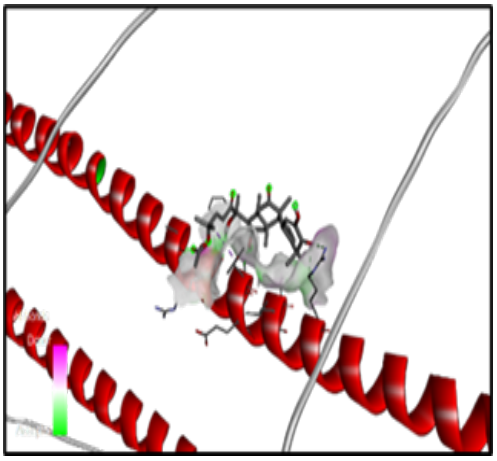


FIGURE 4.15: Analysis of molecular docking of KRT16 protein and Caffeic acid (a) Structure of Human KRT16 protein (b)3D structure of Caffeic acid (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.



(a) (b)



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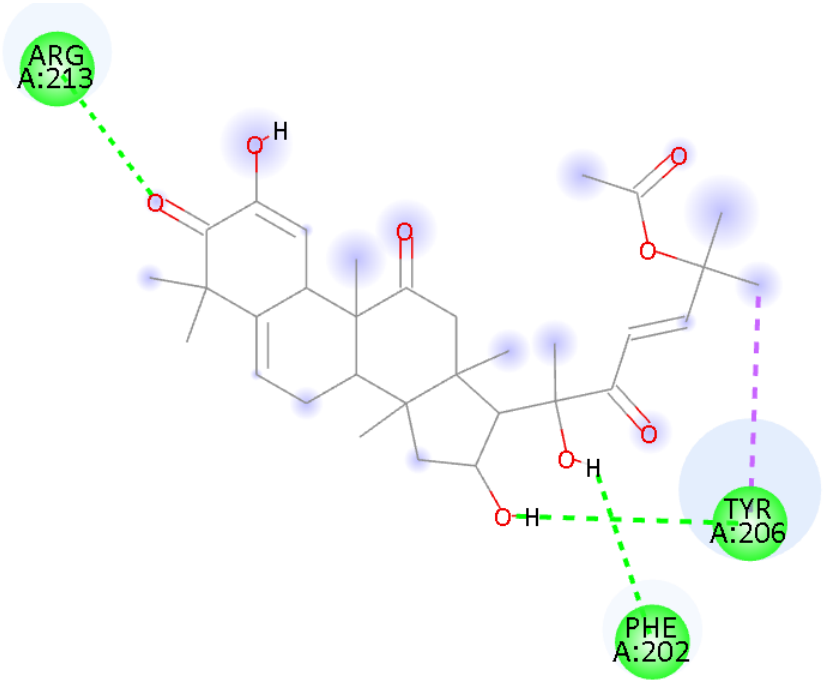


FIGURE 4.16: Analysis of molecular docking of KRT16 protein and Cucurbitacin E (a) Structure of Human KRT16 protein (b)3D structure of Cucurbitacin E (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

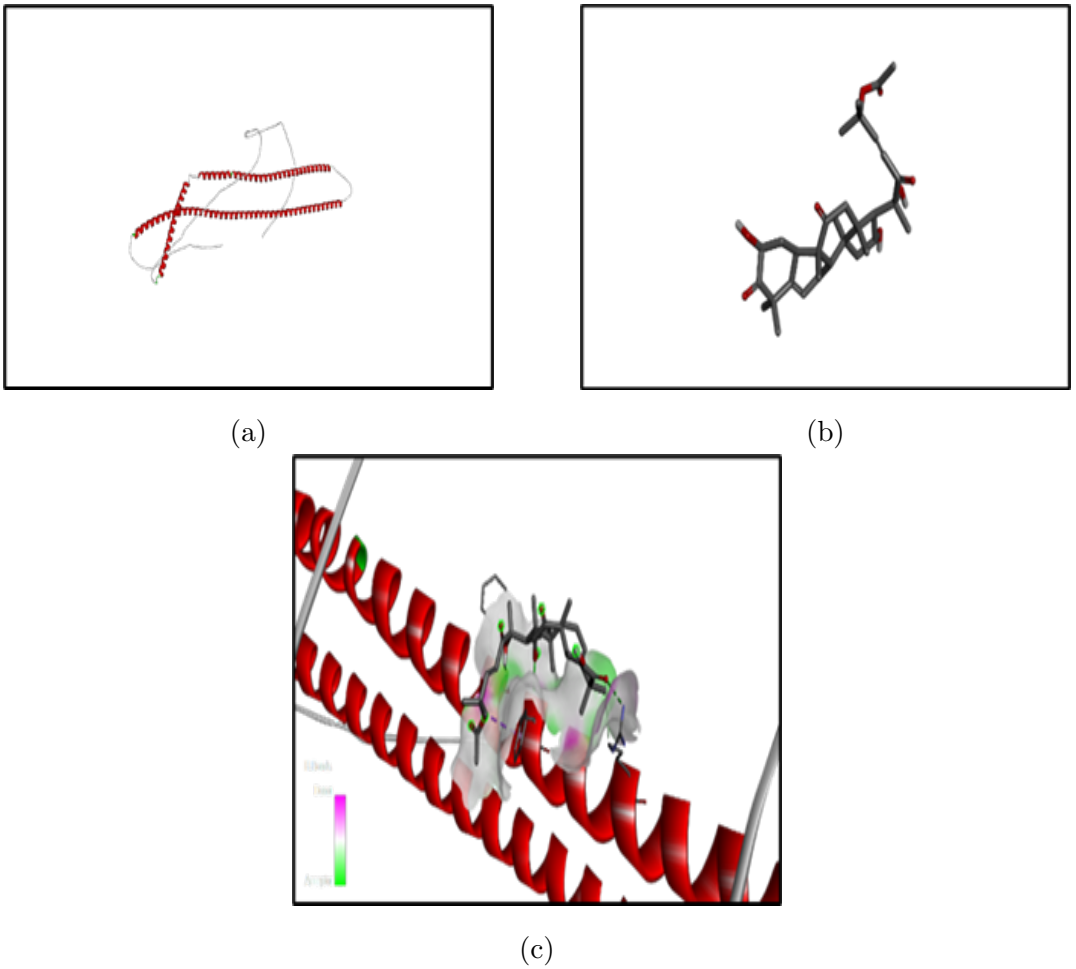


FIGURE 4.17: Analysis of molecular docking of KRT16 protein and Cucurbitacin B (a) Structure of Human KRT16 protein (b)3D structure of Cucurbitacin B (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

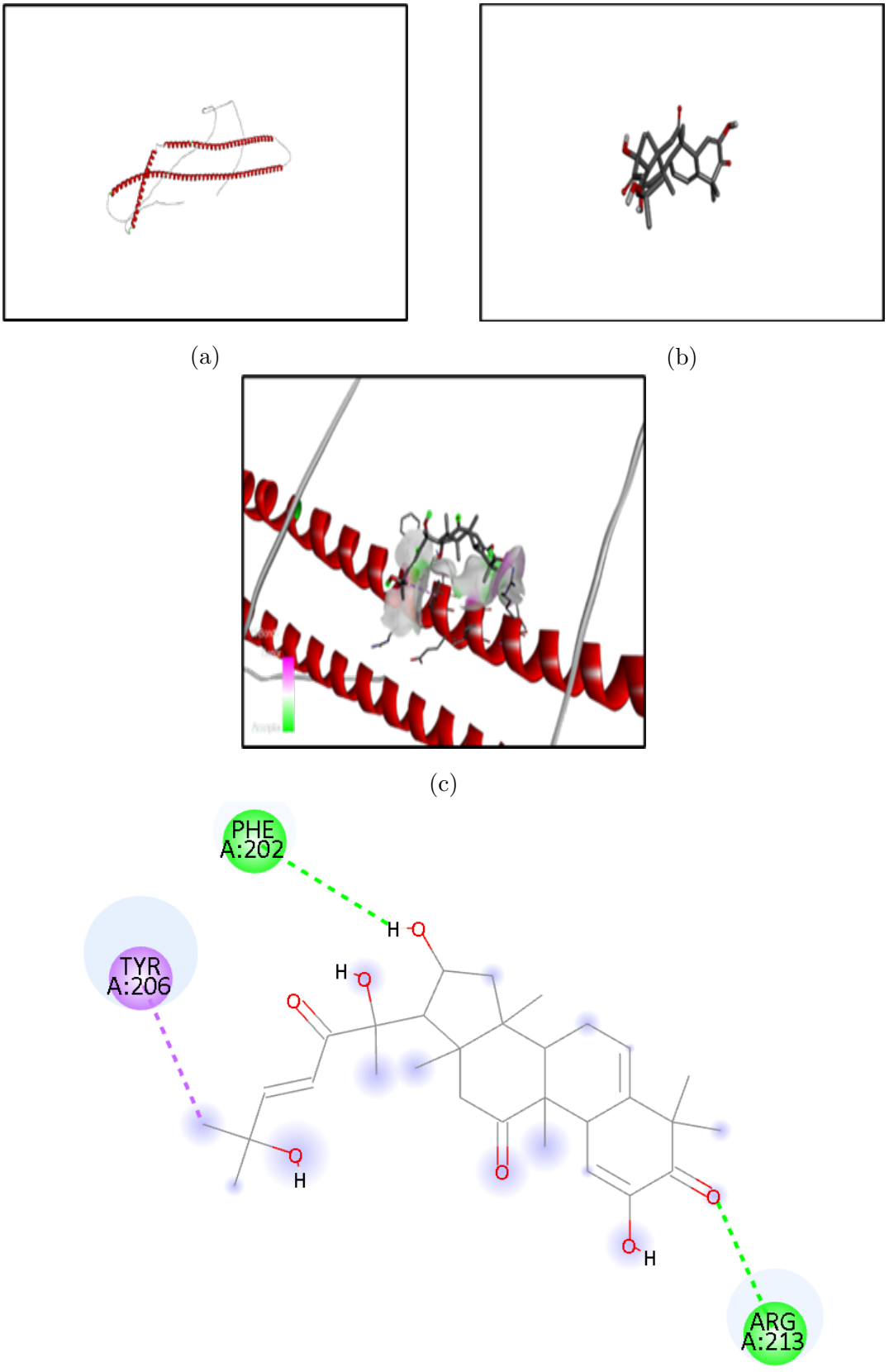
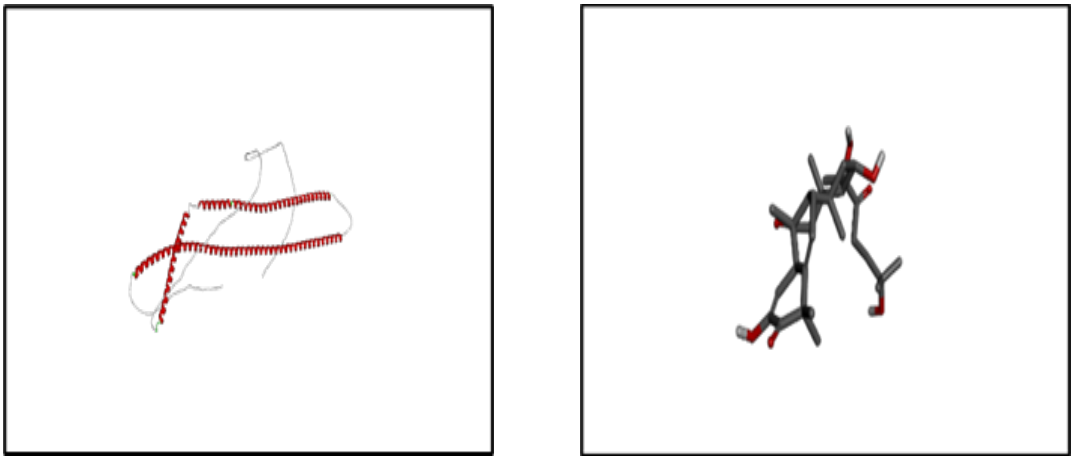
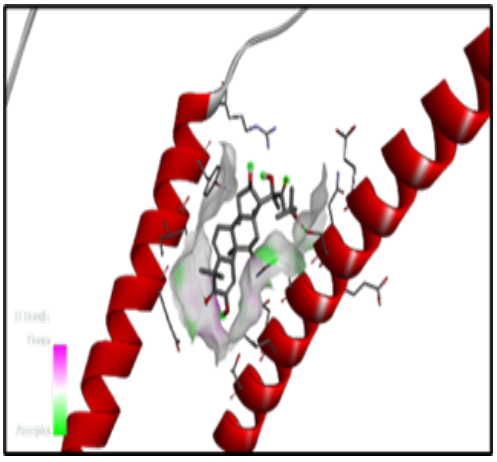


FIGURE 4.18: Analysis of molecular docking of KRT16 protein and Cucurbitacin I (a) Structure of Human KRT16 protein (b) 3D structure of Cucurbitacin I (c) SAS of protein ligand interaction (d) 2D structure of active site of ligand and amino acid residues of protein.



(a) (b)



(c)

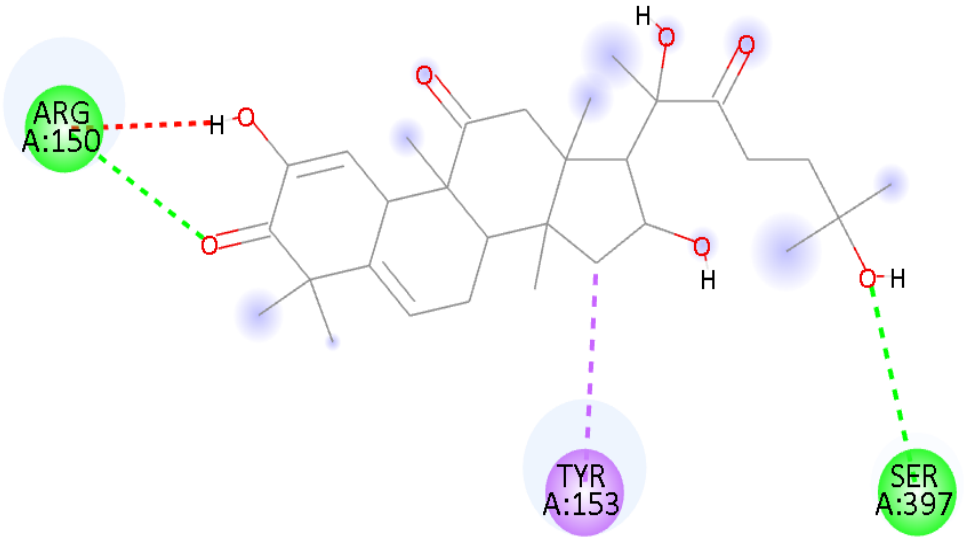


FIGURE 4.19: Analysis of molecular docking of KRT16 protein and Cucurbitacin L (a) Structure of Human KRT16 protein (b)3D structure of Cucurbitacin L (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

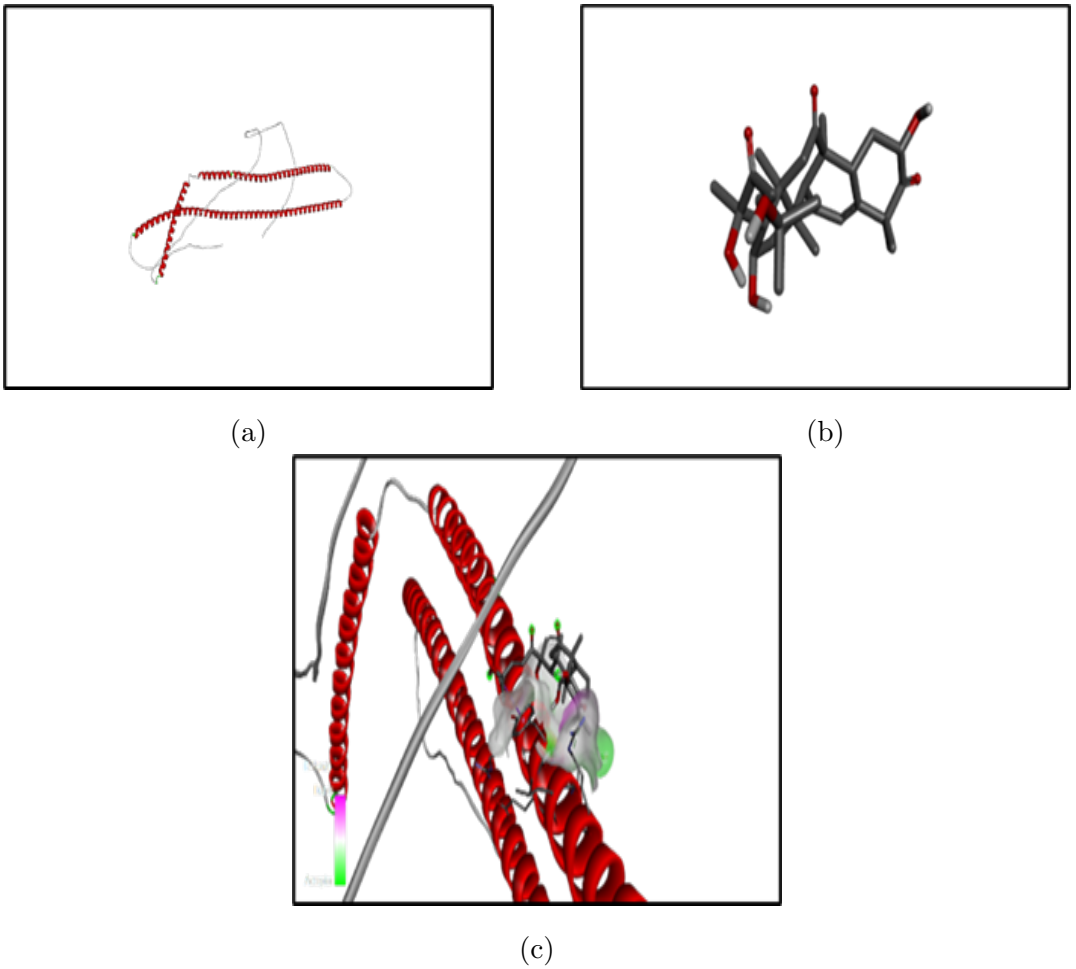


FIGURE 4.20: Analysis of molecular docking of KRT16 protein and Cucurbitacin D (a) Structure of Human KRT16 protein (b) 3D structure of Cucurbitacin D (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

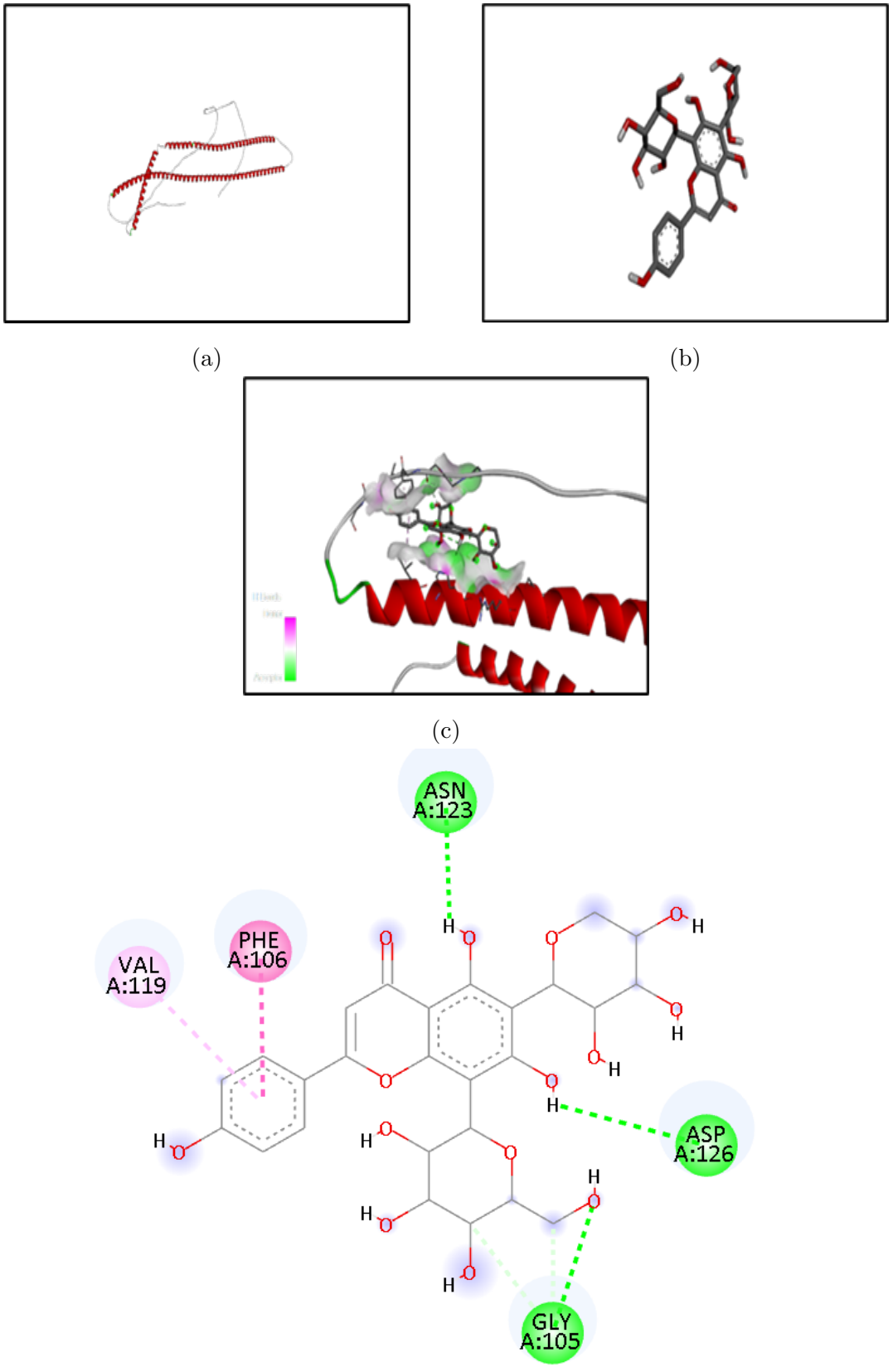


FIGURE 4.21: Analysis of molecular docking of KRT16 protein and Isochaftoside (a) Structure of Human KRT16 protein (b)3D structure of Isochaftoside (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

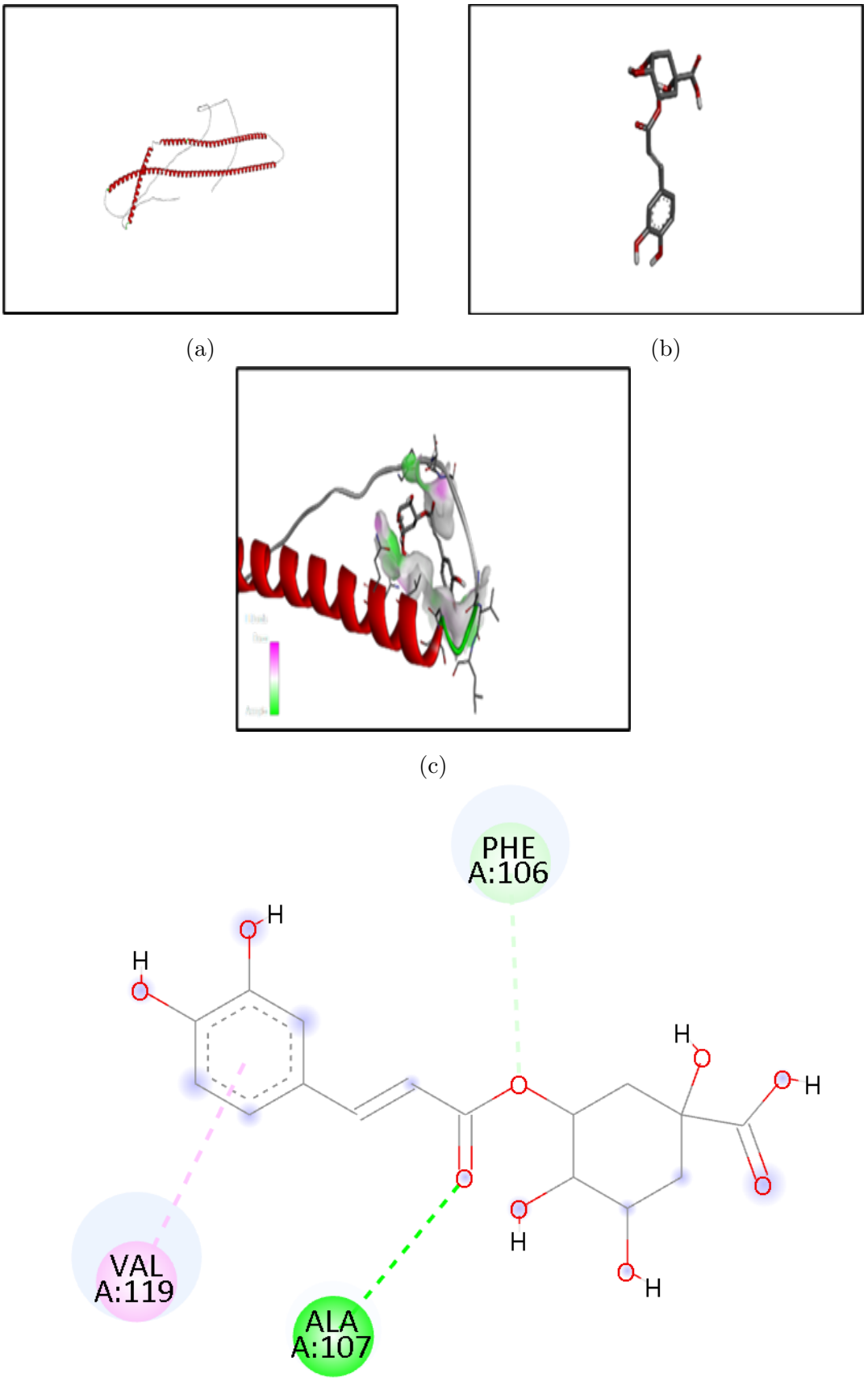


FIGURE 4.22: Analysis of molecular docking of KRT16 protein and Chlorogenic acid (a) Structure of Human KRT16 protein (b)3D structure of Chlorogenic acid (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

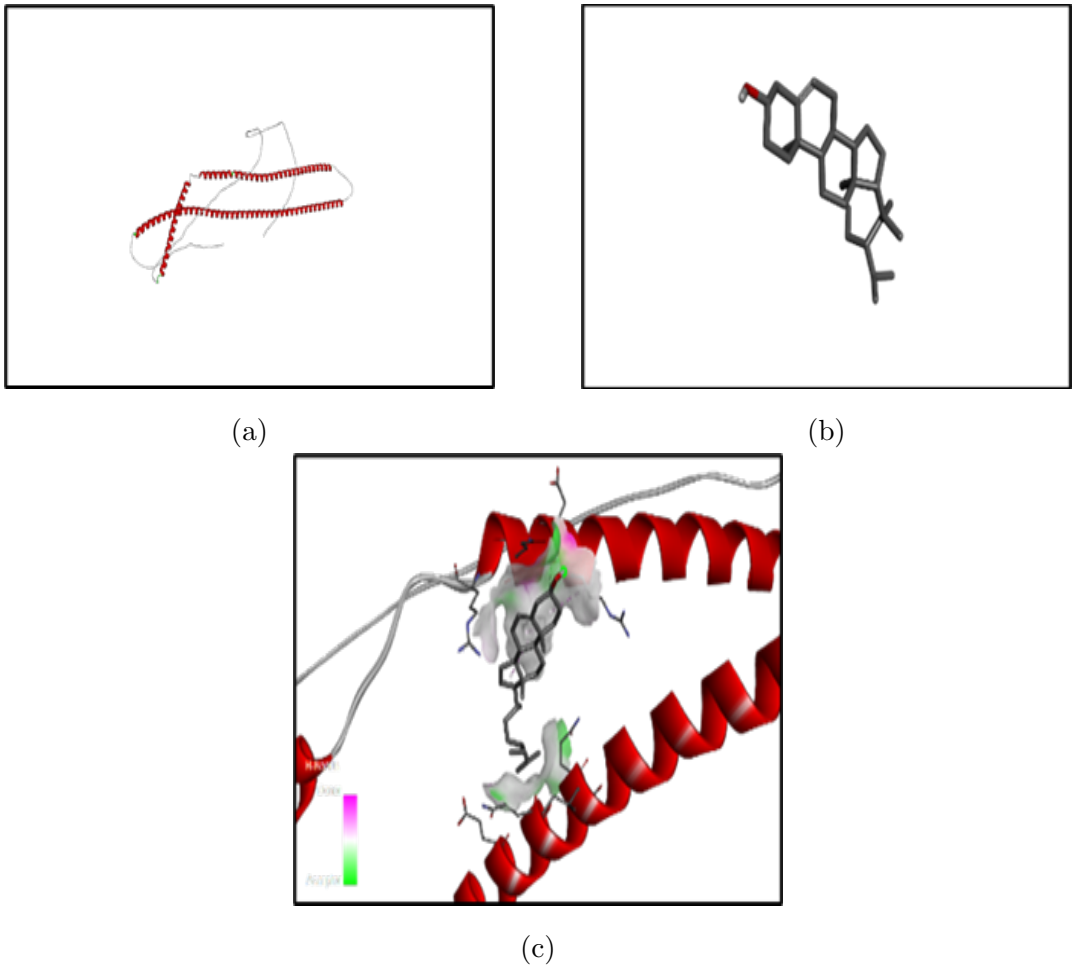
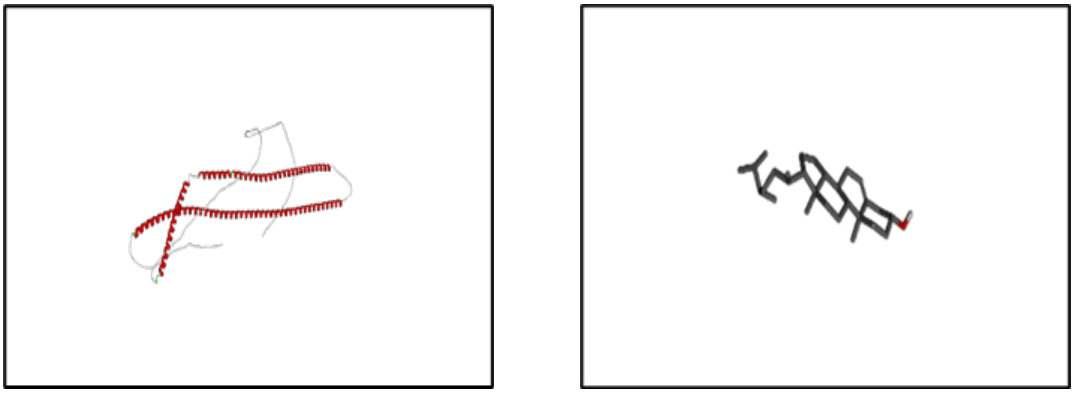
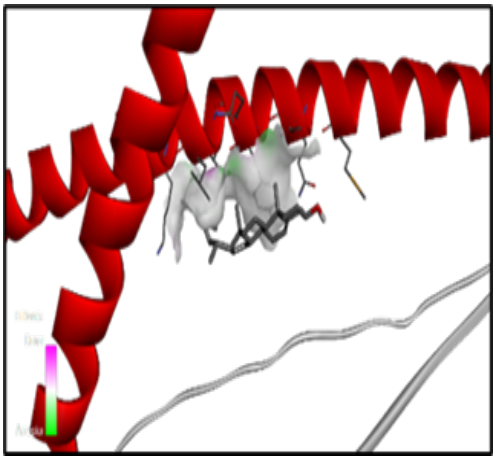


FIGURE 4.23: Analysis of molecular docking of KRT16 protein and B-sitosterol (a) Structure of Human KRT16 protein (b)3D structure of B-sitosterol (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.



(a) (b)



(c)

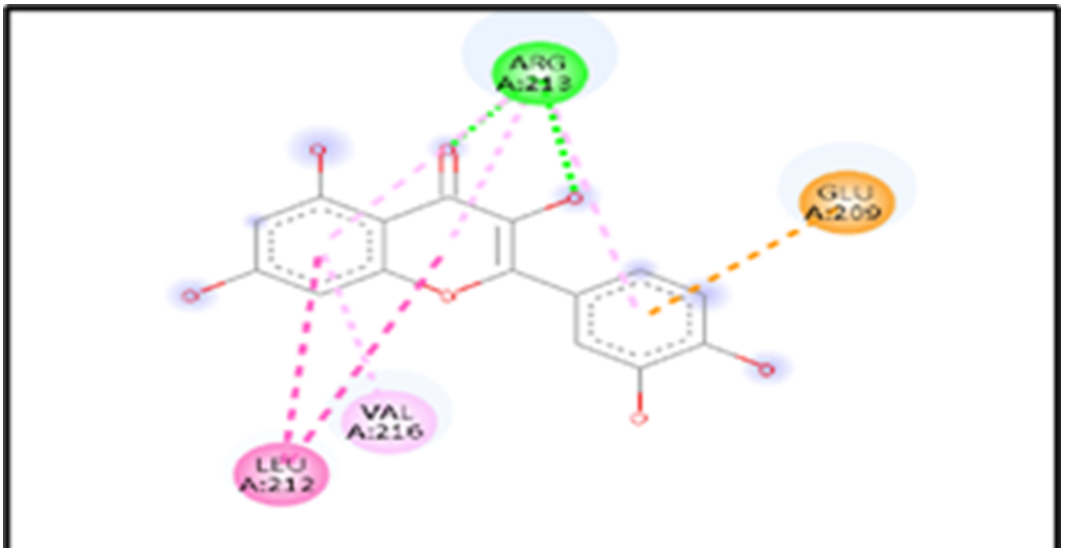
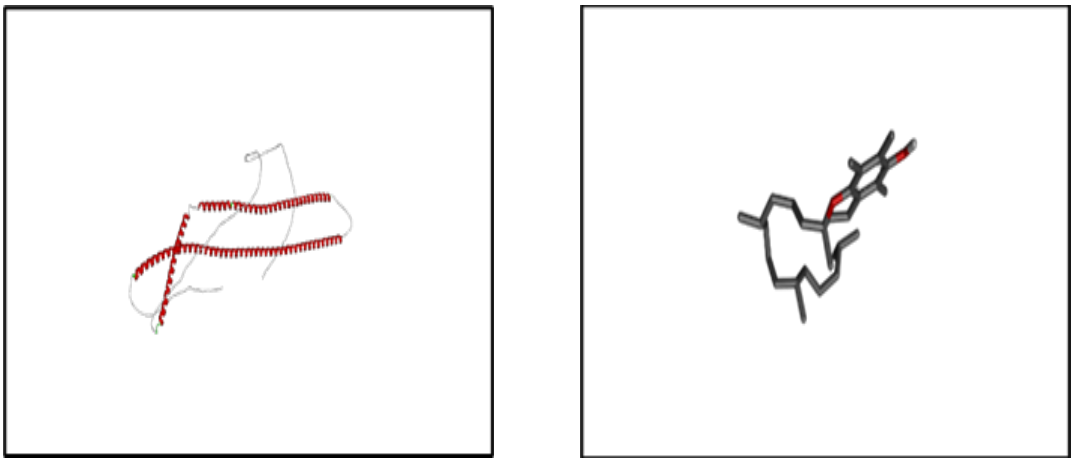
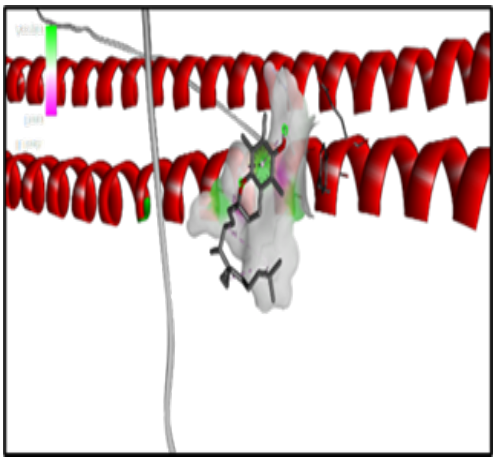


FIGURE 4.24: Analysis of molecular docking of KRT16 protein and Campestrol (a) Structure of Human KRT16 protein (b) 3D structure of Campestrol (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.



(a) (b)



(c)

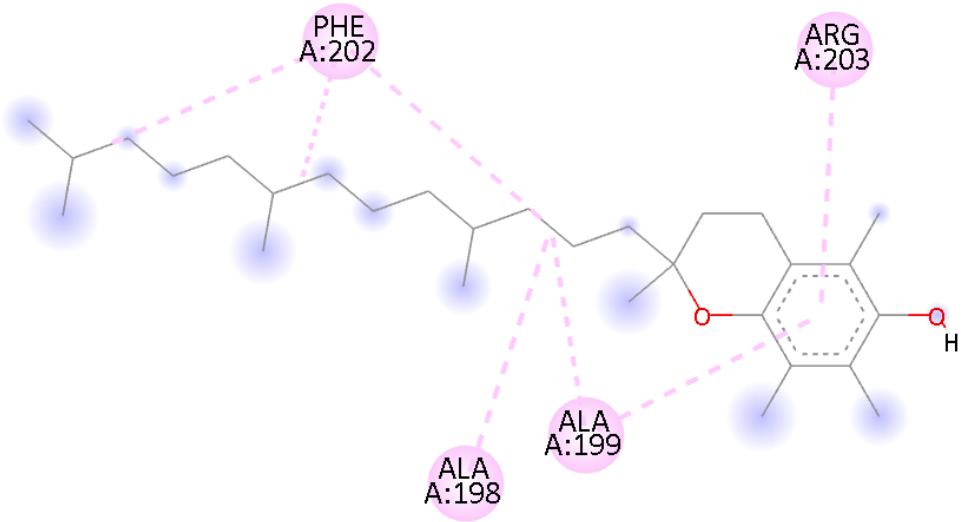


FIGURE 4.25: Analysis of molecular docking of KRT16 protein and Alpha-tocopherol (a) Structure of Human KRT16 protein (b)3D structure of Alpha-tocopherol (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

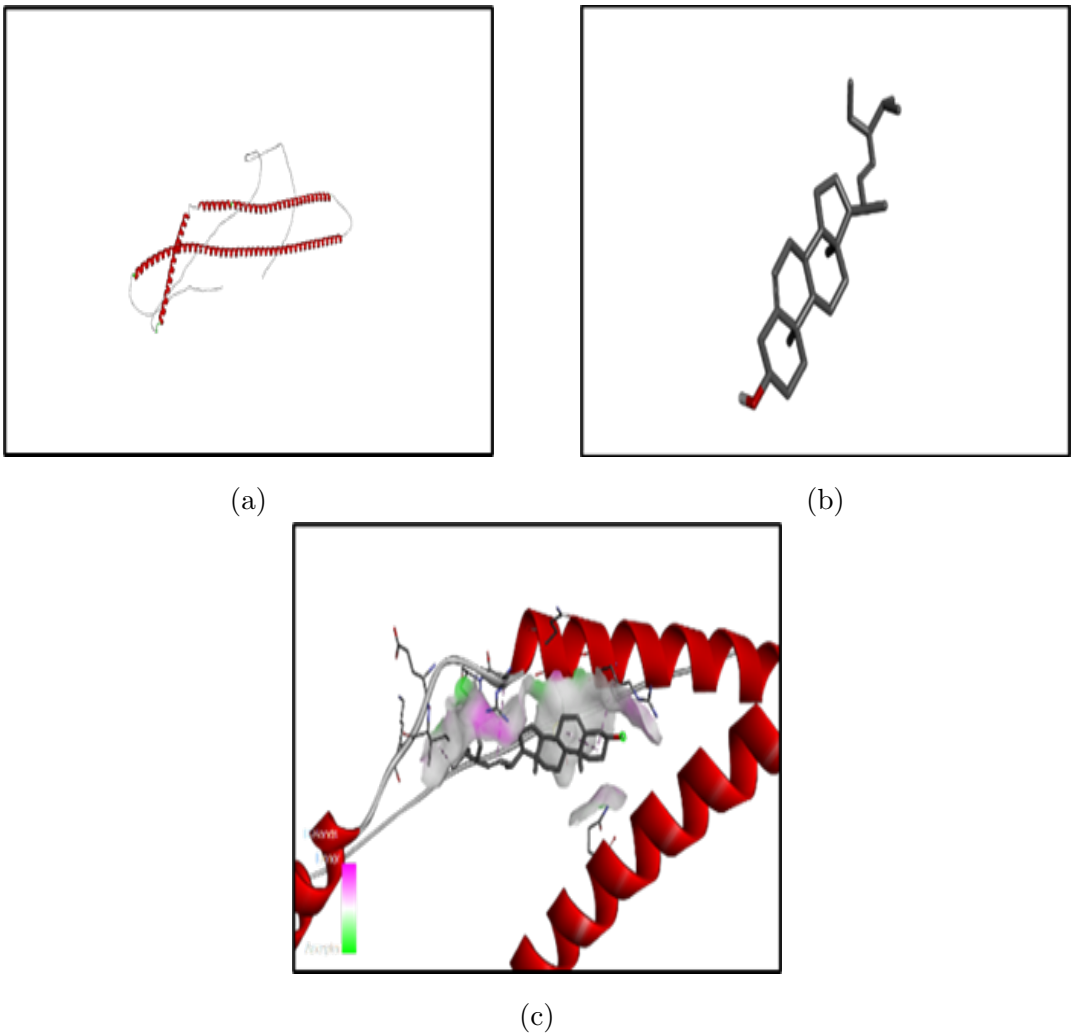


FIGURE 4.26: Analysis of molecular docking of KRT16 protein and Stigmasterol (a) Structure of Human KRT16 protein (b)3D structure of Stigmasterol (c) SAS of protein ligand interaction (d) 2d structure of active site of ligand and amino acid residues of protein.

TABLE 4.9: Docking Results of Selected Compounds with Type of Interactions

Sr No	**Energy Minimization**	**Binding Affinity**	**Rsmd /ub**	**Rsmd /lb**	**Type of Interaction**	**Amino Acid Residues with Bond Distance**
1.	Epicatechin	-5.9	0	0	01. Hydrogen bonds	GLY108; 3.03
						PHE106; 3.46
					02. Hydrophobic bonds	VAL119; 4.83
						VAL119; 5.47
2.	Quercetin	-5.8	0	0	01. Hydrophobic bonds	TYR153; 5.04
						ARG150; 419
						ARG150; 5.15
						ARG150; 5.15
3.	Catechin	-5.7	0	0	01. Hydrogen bonds	ARG150; 2.19
						ARG150; 3.79
					02. Hydrophobic bonds	TYR153; 5.01
						ARG150; 4.89
						ARG150; 5.00
4.	Kaempferol	-5.7	0	0	01. Hydrogen bonds	ARG150; 3.12
						TYR153; 3.04
					02. Hydrophobic bonds	TYR153; 5.36
						ARG150; 4.00
						ARG150; 4.88
5.	P-Coumaric acid	-4.9	0	0	01. Hydrogen bonds	LYS148; 2.20
					02. Hydrophobic bonds	TRP152; 4.26
						TRP152; 3.78
						ILE149; 5.03
6.	Linoleic acid	-3.9	0	0	01. Hydrogen bond	LYS407; 1.99

Table 4.9 continued from previous page

Sr No	Energy Minimization	Binding Affinity	Rsmd /ub	Rsmd /lb	Type of Interaction	Amino Acid Residues with Bond Distance
					02. Hydrophobic bond	LYS407; 4.47
						LYS407; 4.11
						LYS407; 4.00
						LEU403; 4.39
						LEU403; 5.27
						LEU404; 4.58
						LEU403; 5.43
						LEU404; 4.50
						TYR400; 5.13
						TYR400; 4.60
7.	Palmitic acid	-2.8	0	0	01. Hydrogen bond	TYR206; 2.35
					02. Hydrophobic bond	ARG213; 5.01
						ARG213; 4.16
						ARG213; 4,71
						VAL216; 5.32
						VAL216; 4.16
						LEU212; 4.92
8.	Phytol	-3.8	0	0	01. Hydrogen bond	GLN350; 3.08
					02. Hydrophobic bond	LEU347; 5.35
						MET353 ; 5.48
						LYS354; 3.90
						MET353; 3.81
						LEU357; 5.24
9.	Apigenin	-5.5	0	0	01. Hydrogen bond	THR408; 2.73
					02. Electrostatic bond	ARG150; 3.85
					03. Hydrophobic bond	TYR153; 5.36
						ARG150; 4.18

Table 4.9 continued from previous page

Sr No	Energy Minimization	Binding Affinity	Rsmd /ub	Rsmd /lb	Type of Interaction	Amino Acid Residues with Bond Distance
						ARG150; 5.26
						ARG150; 5.24
10.	Luteolin	-6.1	0	0	01. Hydrogen bond	ASP405; 2,66
						THR408; 2.96
						ASP405; 3.64
					02. Electrostatic bond	ARG150; 3.84
					03. Hydrophobic bond	TYR153; 5.43
						ARG150; 4.10
						ARG150; 5.20
						ARG150; 5.24
11.	Ferullic acid	-4.3	0	0	01. Hydrogen bond	ALA107; 3.01
						ASN123; 3.13
					02. Hydrophobic bond	VAL119; 3.54
						PHE106; 5.03
12.	Gallic acid	-3.8	0	0	01. Hydrogen bond	GLN122; 2.07
						ASN123; 2.33
					02. Hydrophobic bond	VAL119; 3.58
13.	Vanillic acid	-3.9	0	0	01. Hydrogen bond	ARG213; 3.03
					02. Hydrophobic bond	ARG213; 3.17
						GLU209; 2.63
						ARG213; 3.58
						VAL216; 5.08
14.	Caffeic acid	-4.4	0	0	01. Hydrogen bond	ALA107; 294
					02. Hydrophobic bond	VAL119; 4.86

Table 4.9 continued from previous page

Sr No	Energy Minimization	Binding Affinity	Rsmd /ub	Rsmd /lb	Type of Interaction	Amino Acid Residues with Bond Distance
15.	Cucurbitacin E	-5.5	0	0	01. Hydrogen bond	TYR206; 3.01
						ARG213; 3.33
						PHE202; 2.57
						ARG213; 3.18
					02. Hydrophobic bond	TYR206; 3.64
16.	Cucurbitacin B	-6.7	0	0	01. Hydrogen bond	ARG150; 3.03
						ARG150; 3.04
						GLN412; 302
						GLU411; 2.24
17.	Cucurbitacin L	-6	0	0	01. Hydrogen bond	ARG150;3.21
						ARG150;3.13
					02. Hydrophobic bond	SER397;3.01
						TYR153; 3.84
18.	Cucurbitacin I	-6.4	0	0	01. Hydrogen bond	ARG213; 3.36
						PHE202; 2.93
					02. Hydrophobic bond	TYR206; 3.79
						ARG203; 3.80
						TYR206; 4.45
19.	Cucurbitacin D	-5.7	0	0	01. Hydrogen bond	PHE202; 2.24
						PHE202; 2.67
					02. Hydrophobic bond	ARG213;3.34
						TYR206; 3.62
20.	Isochaftoside	-6	0	0	01. Hydrogen bond	GLY105; 2.95
						ASP126; 2.30
						ASN23; 2.97

Table 4.9 continued from previous page

Sr No	Energy Minimization	Binding Affinity	Rsmd /ub	Rsmd /lb	Type of Interaction	Amino Acid Residues with Bond Distance
						GLY105; 3.69
					02. Hydrophobic bond	GLY105; 3.57
						PHE106; 3.91
						VAL119; 4.72
21.	Chlorogenic acid	-4.7	0	0	01. Hydrogen bond	ALA107; 3.23
						PHE106; 3.61
					02. Hydrophobic bond	VAL119; 4.54
22.	B-sitosterol	-6.7	0	0	01. Hydrogen bond	ARG150; 3.22
					02. Hydrophobic bond	ARG150; 4.35
						TYR153; 4.81
						TYR153; 5.22
23.	Campestrol	-7.3	0	0	01. Hyrdrogen bond	ARG213; 2.53
						ARG213; 2.18
					02. Electrostatic bonds	GLU209; 4.45
					03. Hydrophobic bond	LEU212; 5.57
						LEU212; 4.85
						ARG213; 3.60
						LEU212; 5.34
						ARG213; 4.54
						VAL216; 4.53
						ARG213; 5.44
24.	Alpha-tocopherol	-5.7	0	0	01. Hydrophobic bond	ALA198; 4.70
						ALA198; 4.25
						PHE202; 5.32
						PHE202; 5.14
						PHE202; 4.16

Table 4.9 continued from previous page

Sr No	Energy Minimization	Binding Affinity	Rsmd /ub	Rsmd /lb	Type of Interaction	Amino Acid Residues with Bond Distance
25.	Stigmasterol	-6.4	0	0	01. Hydrophobic bond	ALA199; 4.97 ARG203; 4.68 ARG150; 5.27 ARG157; 5.22 ILE161; 4.02 TYR153; 4.53 TYR153; 5.10

4.4 Docking Results

4.4.1 Lead Compound Identification

Lipinski's rule, ADMET properties, Binding scores of docking and toxicity prediction were used as primary filter to identify the lead compound. Isochaftoside was knocked out for violating three rules of Lipinski while rest of the 24 ligands still show their drug likeness property.

By screening through ADMET properties, the Cucurbitacins were knocked out because they act as both inhibitors and substrates for P-gp. Cucurbitacin D was highly mutagenic. Quercitin, Kaempferol, Apigenin, and Luteolin were knocked out as they act as CYP1A2 inhibitors. Phytol, B-Sitosterol, Alpha-tocopherol and stigmasterol were knocked out for being acting as hERG inhibitors.

After screening through Binding energies, Ferullic acid,Gallic acid, Vanillic acid, Chlorogenic acid, Palmitic acid, Linoleic acid, P-coumaric acid, Phytol and Caffeic acid were knocked out due to weak binding energies, the suitable lead compound selected is Campestrol as it shows highest binding score of -7.3kcal/mol (form 10 protein-ligand interactions), pass Lipinski rule and also satisfy most of ADMET

parameters, Campestrol just shows negative effect for hepatoxicity so it can be used with little caution.

4.4.2 Simulation

Over 200 nanoseconds, a molecular dynamic simulation analyzed the interaction among a protein receptor and the ligand campesterol. To maintain physiological conditions, the system had a single chain of 473 residues of proteins encircled by specified water molecules and ions. The formula for the ligand campesterol, which is a sterol derivative, is $C_{28}H_{88}O$.

Through the simulation, the overall stability of the protein and ligand was evaluated using the Root Mean Square Deviation (RMSD). The total conformational deviation from the initial structure appears in the protein RMSD. The findings demonstrate that tiny globular proteins may tolerate RMSD variances of 1-3 Å. Sustained protein-ligand interactions indicate that the ligand was still bound. A solid basis for examining binding interactions is provided by the RMSD stabilization about a thermal average, demonstrating that the simulation attained balance.

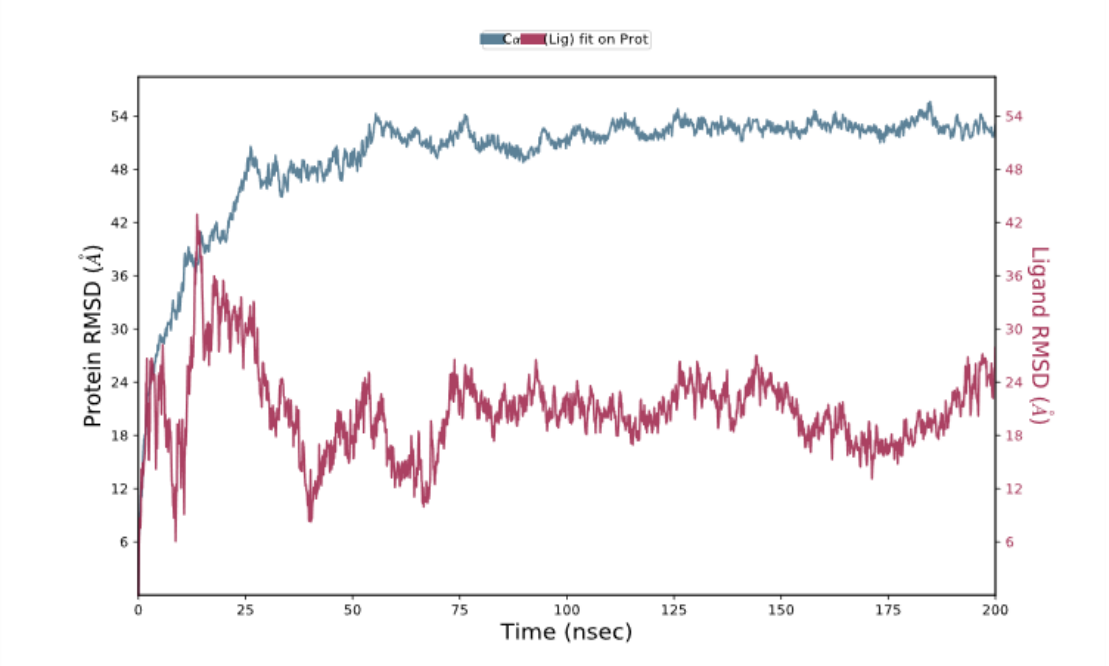


FIGURE 4.27: Analysis of Protein-Ligand Complex RMSD

Per-residue flexibility is measured by protein RMSF, which differentiates between stiff (helices, strands) and movable (loops, termini) areas.

After the protein framework is aligned, RMSD quantifies the movement of the heavy atoms in campesterol for the ligand. When the ligand is well-bound, a steady, low ligand RMSD-usually less than 2.0-2.5 Å—indicates that the ligand stayed firmly in the binding region. As a crucial evidence that campesterol could not separate or experience large-scale displacement throughout the simulation, the paper reveals that the ligand RMSD does not considerably surpass the protein RMSD.

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Higher fluctuation peaks frequently appear in loop portions and the N- and C-terminal sections of the RMSF plot, although secondary structural components like beta-strands and alpha-helices demonstrate less flexibility. With flexible areas perhaps involved in ligand interaction or allosteric regulation, this pattern validates what was expected changing behavior of a folded protein.s

With flexible areas perhaps involved in ligand interaction or allosteric regulation, this pattern validates what was expected changing behavior of a folded protein.

Root Mean Square Deviation (RMSD) has been employed for measuring the protein's stable structure and the ligand's binding efficiency. The plot pattern demonstrates that, as predicted, the greatest peaks are seen in the N- and C-terminal areas, which frequently reach ∼3–6 Åor more in such simulations.

On the other contrary, residues in secondary structure elements (α-helices, β-strands) exhibit much smaller changes, usually between 0.5 and 2.0 Å, indicating their structural stiffness. With RMSF values typically somewhere between 1.5 to 3.5 Å, loop areas showed moderate flexibility.

This pattern emphasizes flexible areas that could be functionally significant while confirming the structural integrity of the protein's core.

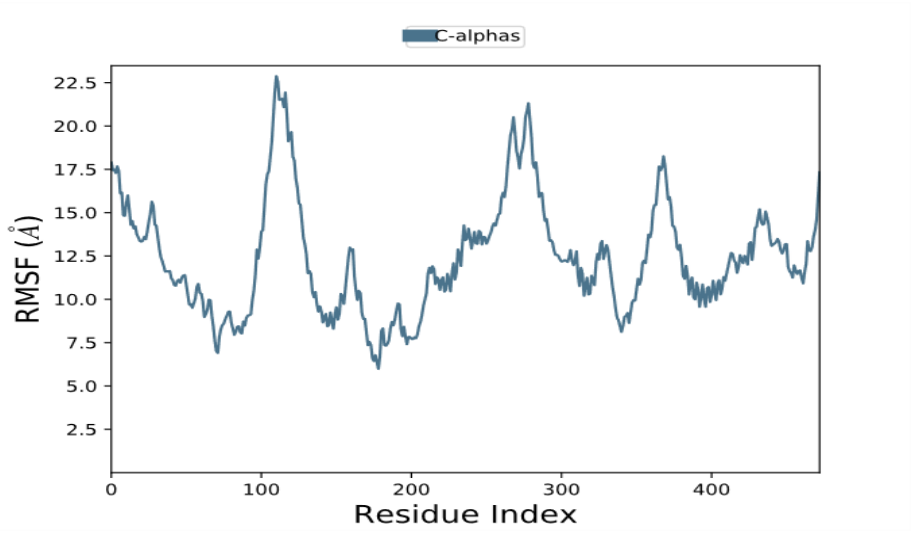

FIGURE 4.28: Analysis of Root Mean Square Fluctuations (RMSF) of Protein (Human KRT16)

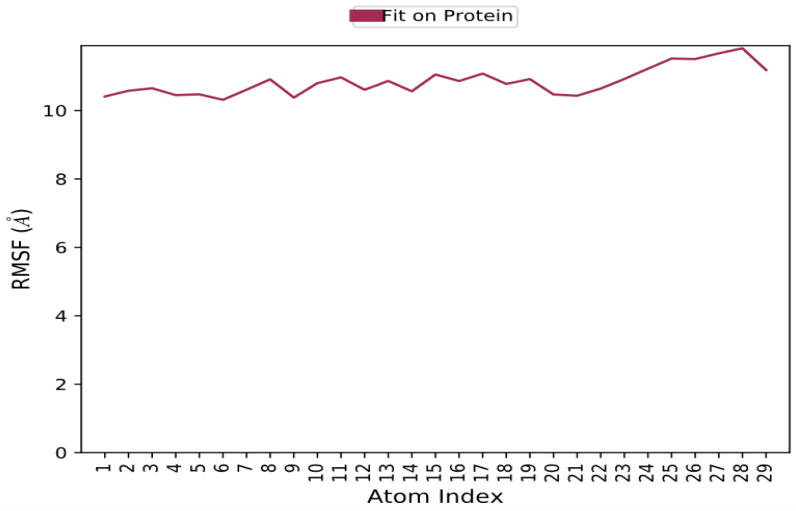

FIGURE 4.29: RMSF of Campestrol on P08779

The ligand RMSF figure shows variation for every atom in campesterol. Atoms in hard ring systems or those that participate directly in protein interactions often exhibit lower RMSF values, which typically range between 0.3 and 1.5 Å. On the other hand, atoms in less tightly packed places or at the end of flexible aliphatic chains could display greater changes, potentially between 1.5 and 4.0 Å. The entropic and enthalpic inputs to binding are guided by this atom-resolved map, assisting in determining which campesterol chemical moieties are more firmly bound by the protein and which maintain more motional freedom.

The continued presence of the protein's secondary structural elements (SSE) indicates that its fold remained extremely stable. The chemical makeup continued to be at 37.03% alpha-helix and 1.32% beta-strand for the duration of the 200 ns simulation, delivering an overall SSE content of 38.35%. This consistency validates the simulation's reliance for examining the native binding pocket configuration by confirming that no significant unfolding or secondary structural restructuring took place.

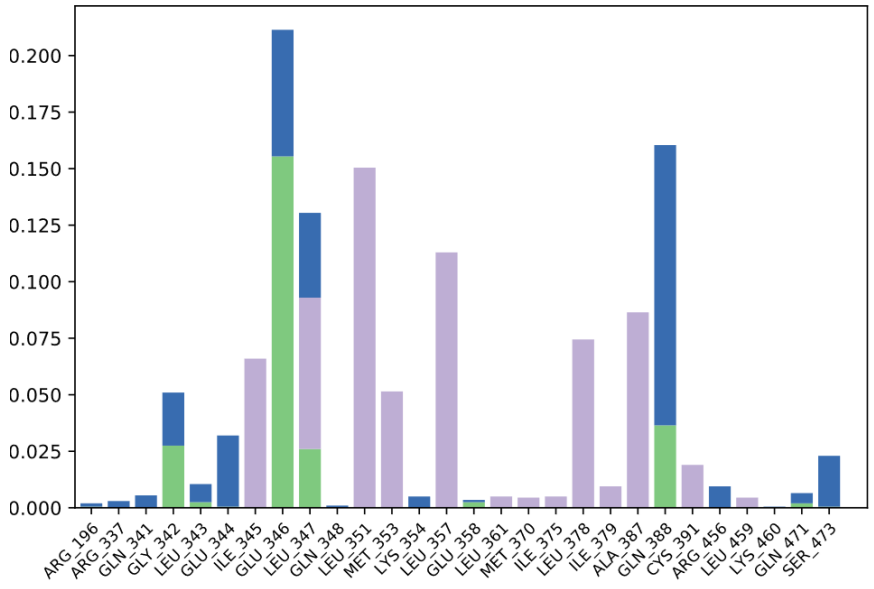

FIGURE 4.30: P08779 and Campestrol Contact

The comprehensive map of continuing protein-ligand interactions is a key feature of our simulation. Four types of contacts were identified and measured: H-Bonds, Hydrophobic, Ionic, and Water Bridges. 28 unique residues continued to have contact with campesterol throughout a sizable portion of the simulation, according to a timing analysis. Among them, ARG_196, ARG_337, GLN_341, LEU_343, GLU_344, ILE_345, and GLU_346 are noteworthy; several of these interactions continued for more than 30% of the simulation duration. These contacts were specified by certain geometric criteria: ionic interactions were determined for opposite charged atoms that were between 3.7 Å, hydrophobic contacts (such as π-π and π-cation) had limit values among 3.6 Åand 4.5 Å, and hydrogen bonds demanded a donor-acceptor distance < 2.5 Å. A complex and stable binding mode is

illustrated by the persistence and complexity of these relationships, which include polar, charged, and non-polar kinds.

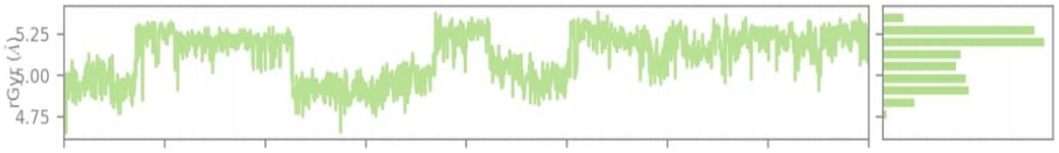

FIGURE 4.31: Radius of Gyration and Docked Complexes (P08779 and Campestrol)

Campesterol's physical and chemical features and its inner structure were also investigated. Dial graphs demonstrated the conformation sampling across time, and the torsion pattern for its six rotatable bonds demonstrated the evolution of dihedral angles. Throughout the simulation, the Radius of Gyration (rGyr), a gauge of the ligand's spatial tightness, was observed. The figure illustrates that the radius of gyration of campesterol changed within a small range of around 4.8 to 5.1 Angstroms, maintaining astonishingly consistent. This minor variation suggests that during the 200 ns simulation while coupled to the protein, the ligand wasn't experiencing considerable compaction or large-scale unfolding, instead maintaining a stable, clearly defined three-dimensional arrangement. This consistency implies that there is not any significant steric strain and that the bound configuration is similar to native.

The surface area that a water molecule may reach, known as the Solvent Accessible Surface Area (SASA), was found to be around 683.5 $Å^2$. Since SASA takes into consideration the probe rolling across the van der Waals surface, which results in a bigger area, the notable discrepancy between MoISA and SASA is predicted. As is common for a ligand in a tightly packed binding pocket, the stability of the SASA value over time confirms that the ligand's exposure to solvent remained constant.

Polar Surface Area (PSA): The PSA, or the fraction of the SASA given just by oxygen and nitrogen atoms, has been shown to be approximately 37.8 $Å^2$, which helps to comprehend the polarity of the ligand. This relatively small PSA is in line with the sterol-based, mostly hydrophobic structure of campesterol, which has a single hydroxyl group as its main polar constituent. The numerous hydrophobic

contacts found with residues like LEU_343, ILE_345, and LEU_357 show that hydrophobic interactions play an important role in the binding process, which has been verified by the consistently low PSA value.

In the simulation process, no intramolecular hydrogen bonds (intraHB) were observed in the campesterol ligand. This result verifies the ligand's conformational analysis, illustrating that the shape of the protein's binding pocket and its inner stiffness of its fused ring system determine the ligand's stable bound condition as opposed to internal H-bonding connections.

Campesterol maintains a particular, low-strain conformation inside a complementary binding pocket, predominantly through hydrophobic complementarity and specific polar contacts, according to these metrics, which are constant over time and typical of a hydrophobic molecule.

This simulation offers substantial evidence that campesterol and the target protein form an eternally distinct mixture. A network of interactions involving important residues, including hydrogen bonding, hydrophobic wrapping, ionic forces, and water-dependent bridges, enable its binding.

Chapter 5

Discussion

One of the most common dermatological disorders in the world, Acne vulgaris , a chronic inflammatory infection of the pilosebaceous unit marked by atypical keratinization, high sebum synthesis, microbial colonization, and inflammatory reactions [224]. The defining characteristics of acne pathogenesis, increased number of keratinocytes and follicular blockage, have been closely related to the altered expression of keratin proteins, especially KRT16 [225]. KRT16 overexpression exacerbates acne lesions and delays skin healing processes by promoting aberrant differentiation of the epidermal cells and elevated inflammatory signalling [226]. There is a dire requirement for more efficient and secure alternatives because current anti-acne treatments, such as retinoids, antibiotics, and hormonal drugs, frequently result in side effects include dermal irritation, resistant to antibiotics, and systemic toxicity [227]. *C. colocynthis* comprises sterols, triterpenoids, flavonoids, alkaloids, and with reported anti-inflammatory and antibacterial properties, as demonstrated by phytochemical profiling [228]. Campesterol was determined to be the lead molecule among the tested phytochemicals because of its excellent molecular docking score, steady binding conformation, along with suitable drug-likeness characteristics. Since campesterol is a naturally found phytosterol that has structural similarities with cholesterol, it might interact successfully with structural and membrane-associated proteins such keratins [229]. According to earlier studies, campesterol greatly decreases inflammation by minimizing oxidative stress and

suppressing pro-inflammatory cytokines [230]. Campesterol's significant binding affinity for KRT16 has been established by molecular docking studies, indicating a possible function for it in regulating the hyperproliferation of keratinocytes linked to acne [231]. Hydrogen and hydrophobic bonds with important amino acid residues necessary for structural stability promoted the stable association of Campesterol inside the active region of KRT16 [232]. Restoring normal epidermal proliferation and preventing inappropriate keratin aggregation depend on these molecular connections [233].

Campesterol's hydrophobic sterol backbone greatly contributes to its binding stability inside the protein pocket of KRT16, according to structure–activity relationship research. Hydroxyl functional groups promote complex durability and ligand-protein affinity by improving hydrogen bonding interactions [234]. These results are in line with other studies showing the significance of sterol functional groups in anti-inflammatory action and protein regulation. Campesterol meets the necessary criteria for oral bioavailability, such as molecular weight, hydrogen bond donors, and acceptors, leading to a drug-likeness evaluation based on Lipinski's rule of five. Phytosterols' beneficial physical and chemical properties encourage more formulation research for dermatological usage, though they are mainly thought of for topical application. Campesterol's ability as a safe and efficient anti-acne treatment has been enhanced by its acceptable drug-likeness profile. This study's in-silico method decreases the time, expense, and ethical issues related to experimental research while offering insightful information on the molecular pathway of campesterol towards KRT16[235]. Prior to in-vitro and in-vivo validation, computational modelling established itself as a solid initial strategy of discovering lead compounds and estimating their therapeutic value [236].

Adapalene serves as a third-generation dermal retinoid that is frequently utilized as modern-day acne treatment because of its potent comedolytic and anti-inflammatory effects along with its distinctive affinities for retinoic acid receptors. The main pathogenic phase in the formation of acne, microcomedone production, is avoided, follicular hyperkeratinization is decreased, and keratinocyte differentiation is adjusted. Adapalene should be used for long-term clinical usage since

it exhibits better photostability and a lesser likelihood for irritation than recent retinoids. By regulating gene expression involved in inflammatory pathways and cell proliferation, its molecular mechanism targets the structural and inflammation elements of acne lesions. Adapalene serves as a helpful standard for assessing the curative properties of new plant-derived compounds like campesterol and is therefore regarded as a standard reference chemical in anti-acne research.

This work is constrained by the fact that it depends on computer analysis, which was unable to precisely represent complicated biological systems, despite the encouraging results [237]. The inhibiting impact of campesterol on KRT16 expression and acne-associated irritation require experimental confirmation employing cell-based assays and animal models [238]. To determine its therapeutic efficiency and safety, future research ought to concentrate on in-vitro keratinocyte models, topical formulations based on campesterol, and clinical assessment [239]. Overall, the current examination shows that campesterol from *C. colocynthis* is possible plant-derived lead chemical that targets KRT16, delivering a new and safer method of treating acne.Keratin-16's established involvement in stress-induced epidermal processes and hyperproliferative skin disorders strongly supports its choice as a molecular target in the acne investigation [240]. Increased expression of Keratin-16 is often linked to excessive cornification and abnormal keratinocyte differentiation, which may lead to follicular obstruction and inflammatory cascades [241]. Therefore, rather than only treating surface symptoms of acne, modulating Keratin-16 is a logical therapeutic approach that attacks acne at the molecular and structural level [242].

Due to their capacity to restore epidermal equilibrium without causing the cytotoxic effects frequently seen with synthetic medications, the combination of plant-derived chemicals involving keratin proteins has received more attention [243]. In particular, natural sterols have shown favorable effects on cellular homeostasis, inflammatory control, and skin barrier regulation, making them good candidates for dermatological purposes [244]. The primary chemical found in this investigation, campesterol, showed a binding orientation which promotes stable accommodation across the Keratin-16 binding area, showing that it may disrupt aberrant keratin

assembly [245]. By normalizing keratin filament organization, such interference may lessen hyperkeratinization and comedone creation linked to the advancement of acne [246].

Campesterol could potentially be able maintain a lengthy contact with KRT16, which is necessary for proper biological management, according to the molecular stability seen during docking research [247]. By minimizing dissociation rates and boosting functional effect at the target region, stable ligand-protein complexes have been reported to improve therapeutic efficacy [248]. Campesterol's general physicochemical characteristics, in addition to its target-specific binding, demonstrate its eligibility for dermatological usage, especially in topical medications intended for chronic skin disorders like acne [249].

It has been well documented that phytosterols integrate effectively in lipid-rich settings such as the stratum corneum, improving skin permeability as well as localized action [250]. Campesterol's potential as a lead molecule is further strengthened by its attractive drug-likeness profile, as molecules with acceptable physicochemical properties are far more likely to successfully advance through subsequent stages of therapeutic development [251]. Early detection of these characteristics using computational screening lowers attrition rates and boosts trust in lead optimization techniques [252]. In a larger sense, the usage of *C. colocynthis* considered as medicinal plant coincides with the growing curiosity in evidence-based certification of traditional treatments across the world [253]. Finding new bioactive compounds becomes simpler and scientific credibility is increased when ethnopharmacological expertise is integrated with sophisticated computational techniques [254].

The research's in-silico architecture provides a scientific and repeatable way to assess plant-derived chemicals against therapeutically significant targets [255]. These approaches are becoming progressively recognized as crucial preparatory equipment that direct experimental research and save needless laboratory costs [256]. It is crucial to recognize that biological systems are intrinsically complicated and may react differently under physiological settings, even if the computational results offer solid theoretical support [256].

Overall, the results of this study provide support to *C. colocynthis* potential as a beneficial natural source for the creation of safer anti-acne medications. A logical and valid scientific approach is provided by the evolution from recognizing the pathophysiology of acne to picking KRT16 as a molecular target and then identifying Campesterol as the lead chemical. Campesterol's capacity to regulate aberrant keratinocyte proliferation, a crucial component in the formation of acne, is suggested by the strong binding relationship between it and Keratin-16. Additionally, Campesterol's good drug-likeness profile enhances its eligibility of further pharmacological investigation.

Chapter 6

Conclusion and Future Recommendations

6.1 Conclusion

One of the most severe chronic inflammatory skin diseases harming adults and adolescents around the globe is acne vulgaris. It puts a heavy social, psychological, and physical load that can often result in permanent scars, mental discomfort, and low self-esteem. Even though there are many treatment choices, long-term use of traditional anti-acne medications is linked to side effects and rising resistance, underscoring the critical need for more reliable and robust substitutes on a worldwide scale. Because of their multitargeted effects and decreased toxicity, medicinal plants high in biologically active phytochemicals have become viable sources for novel acne treatments.

Using an integrated in silico method, the current work aimed to investigate the anti-acne potential of bioactive chemicals created by *C. colocynthis* (bitter apple). The main goal was to find promising phytochemicals that could interact with a chosen target protein linked to acne and assess their efficacy and drug-likeness by using computational analysis.

In order to reach the first objective, phytochemicals found from *C. colocynthis* have been found after a thorough review of the literature. A total of twenty-five bioactive chemicals were chosen as a result of this methodical screening. These phytochemicals' chemical structures were obtained from PubChem and made ready for additional computer analysis. This stage created a solid basis for further virtual screening investigations by confirming a choice of structurally varied and physiologically significant ligands.

The identification and development of the target protein linked to acne etiology was the study's second objective. The AlphaFold database, providing highly exact protein models utilizing deep learning-based predictions, provided a three-dimensional model of the selected protein. To make sure the recovered structure was suitable to conduct molecular docking and simulation research, it was thoroughly analyzed and processed. The application of AlphaFold improved the accuracy of structure-based drug design and addressed the problem of unavailable experimentally proven structures.

Molecular docking along with virtual screening were used to determine the chosen phytochemicals' binding affinity with the target protein in order to achieve the third goal. Schrödinger was used for docking analysis, allowing for a thorough assessment of ligand-protein interactions. Key intermolecular interactions, binding poses, and docking scores were used to evaluate the docked complexes. Additionally, drug-likeness assessment and ADME profiling were carried out, utilizing Lipinski's Rule of Five. With just a small number of compounds exhibiting minor infractions, the results showed that most screened compounds had excellent pharmacokinetic properties, confirming that most ligands had acceptable drug-like characteristic.

The fourth objective was to evaluate the stable and dynamic behavior of the most promising ligand–protein complex via molecular dynamics (MD) simulation studies. Its structural adaptability, conformational reliability, and interaction permanence over time were shown using MD simulations. The chosen complexes were stable over the simulation time, according to the examined characteristics, which included hydrogen bond stability, root mean square deviation (RMSD), and root

mean square fluctuation (RMSF). These results supported the prospective use of the newly identified phytochemicals to be effective anti-acne medicines and further verified the docking results.

In order to wrap up, our study's integrated in silico approach effectively isolated possible bioactive compounds from *C. colocynthis* with desirable drug-likeness, major binding affinity, and stable dynamic behavior against the chosen target protein associated with acne. The results support bitter apple's therapeutic value as a potential natural source for the production new anti-acne medicines.

6.2 Future Recommendations

Even though the current work offers insightful computational information, further study is recommended to back up and verify the results. The biological activity, security, and effectiveness of the newly identified lead compounds should be verified by experimental in vitro along with in vivo studies. In order to validate the hypothesized connections, enzyme inhibition experiments and cell-based investigations utilizing models relevant to acne would be very helpful.

Moreover, to evaluate these drugs' appropriateness for topical formulations, enhanced pharmacokinetic and toxicity analysis, involving metabolism and skin penetration tests, is advised. To improve binding affinity and drug-likeness, optimized structure and lead modification techniques may also be used.

In order to further clarify the polypharmacological nature of *Citrullus colocynthis*, future research might broaden this study by investigating additional molecular targets related to acne and using multi-target docking approaches. The developing of safe, efficient, and plant-based anti-acne remedies will eventually become easier by combining computational projections with experimental confirmation.

The results of this study may also provide a framework for the logical development of plant-based anti-acne treatments. Future formulation research can make use of the found lead chemicals from *Citrullus colocynthis*, especially for the creation of topical gels, creams, or nano-delivery methods meant to enhance skin penetration

and therapeutic efficacy. The translation of these insights into useful dermatological applications may be accelerated by integrating computer predictions with formulations science and clinical examination. Thus, the current in silico study not only advances our knowledge of the interactions between natural compounds and proteins in the treatment of acne, but it also offers a systematic strategy to the creation of secure, successful, and permanent anti-acne therapies.

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