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EVALUATION OF BIOACTIVE COMPOUNDS FROM *Tribulus terrestris* TO EXPLORE ITS POTENTIAL IMPACT ON KIDNEY STONES

Mehaboob Roshini. H[1*], Santhosh.R[1], Sasidaran.V[2], Neraneeyan Suresh[3]

Department of Biotechnology, Bharath Institute of Higher Education and Research, Chennai 73

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Abstract

This project report involves the study of evaluation of bioactive compounds extracted from *Tribulus terrestris* and their potential impact on kidney stones. The study encompassed Soxhlet extraction using methanol as a solvent to obtain the plant extract, subsequent phytochemical analysis, and assessment of antioxidant properties through DPPH assay radical scavenging. GCMS analysis identified and characterized compounds within the extract, while molecular docking techniques elucidated interactions between the identified compounds and key components in kidney stone formation. Phytol was identified to be the most suitable compound among the compounds characterized. Molecular dynamics simulations explored the dynamic behaviour of the compounds, including the protein-ligand complex involving Chain A of 2RDU protein and the ligand UNL. The results offer valuable insights into the therapeutic potential of specific compounds from *Tribulus terrestris* in addressing kidney stones.

Key Words: *Tribulus terrestris*, kidney stones, GCMS, molecular docking, molecular dynamics.

INTRODUCTION

Urolithiasis, commonly known as kidney stones, is a prevalent issue in the urinary tract that results from the accumulation of rigid crystals formed by mineral or salt build-ups. This condition affects 2-20% of the global population, causing pain and obstruction of urine flow, and poses a significant societal cost owing to its widespread occurrence and high recurrence rates (Johri et al., 2010). The term "urolith" originates from the Greek words "ouron" meaning urine and "lithos," meaning stone (Osborne et al., 1999). Various types of renal stones are primarily composed of calcium oxalate hydrates,

ammonium magnesium phosphate, calcium phosphates, uric acid, urates, cystine, and xanthine (Grases et al., 1998). Stones > 5 mm require medical intervention, including increased fluid intake, thiazides, allopurinol, potassium citrate, diuretics, and surgical procedures such as extracorporeal shock wave lithotripsy (ESWL), ureterorenoscopy (URS), percutaneous nephrolithotomy (PCNL), or a combination of these techniques. Despite technological advancements, kidney stone treatments have limitations such as serious side effects and incomplete prevention of recurrence. Issues such as shock wave trauma, residual fragments post-ESWL, infection risks, and long-term medical effects emphasize the need for alternative approaches.

Some of the treatment options available for kidney stones are as follows:
1. Extracorporeal Shock Wave Lithotripsy (ESWL) is a noninvasive method that uses shock waves to break stones into smaller fragments.

2. Ureteroscopy is a minimally invasive procedure, in which a small scope is inserted through the urethra and bladder to reach the ureter or kidney for stone removal or fragmentation.

3. Percutaneous Nephrolithotomy (PCNL) is another minimally invasive procedure in which a small incision is made in the back to directly access the kidney for stone removal.

4. Medical Expulsive Therapy (MET) involves the use of medications to help pass smaller stones, such as alpha-blockers, and relax the ureter.

Preventive measures for kidney stones involve ensuring adequate fluid intake to maintain a daily urine output of at least 2 litres, making dietary modifications based on individual metabolic abnormalities, and utilizing medications for prevention of stone recurrence. The relapse rate for secondary stone formation is estimated to be 10-23% per year, 50% in 5-10 years, and 75% in 20 years. Individuals with a history of kidney stones are at increased risk of developing chronic kidney disease, end-stage renal failure, cardiovascular disease, diabetes, and hypertension. The formation of kidney stones is a complex process involving the nucleation, growth, aggregation, and retention of urinary stone constituents within tubular cells. Currently, there are no satisfactory drugs available for stone dissolution, leading to reliance on herbal medicines that are known for their efficacy and fewer side effects in reducing renal stone recurrence.

Kidney stones are formed when urinary compounds solidify, causing severe pain and urinary blockage. Adequate fluid intake, dietary adjustments, and herbal remedies are all effective preventive measures. Urolithiasis is a condition in which stones pass through the renal pelvis to the ureter, bladder, and urethra. In Malaysia, 10-20% of the population experience kidney stones, with calcium stones being the most common. Stones such as uric acid, struvite, and cystine pose various risks. The formation of calcium oxalate or calcium phosphate stones is influenced by factors, such as oxalate intake. The symptoms of kidney stones include body pain, dysuria, hematuria, cloudy urine, frequent urination, nausea, vomiting, fever, and chills. The narrow urinary duct makes the passage of large stones extremely painful, potentially damaging the ureteral wall as they move through the tract. Despite the significant advancements in modern medicine, the use of herbal remedies remains widespread.

Herbal medicines have a long history, dating back to ancient times, and are employed for general health maintenance and the treatment of specific ailments. Traditional medicine systems have extensively incorporated herbal treatments for a diverse range of diseases. Globally, over 35,000 plant species are used in various human cultures for medicinal purposes. The World Health Organization (WHO) highlights that 80% of the world's population primarily relies on traditional medicine, with a significant portion of traditional therapies involving the utilization of plant extracts or their active constituents.

In recent years, there has been growing interest in the extraction of phytochemicals from various plant sources because of their potential therapeutic benefits. Phytochemicals, also known as biologically active compounds, are organic compounds found in plants and possess various medicinal properties. These compounds have been extensively studied for their potential in the treatment and prevention of various diseases including cancer, cardiovascular disorders, and kidney stones. One such plant that has gained attention in the field of phytochemical extraction is *Tribulus terrestris*. *Tribulus terrestris*, commonly known as puncture vine or gokshura, is a medicinal plant that belongs to the Zygophenaceae family and is widely distributed in various regions of the world, including India, China, and the Mediterranean. The plant has been used for centuries in traditional medicine, including Ayurveda and Traditional Chinese Medicine, to treat various health conditions, particularly those related to genitourinary disorders.

Studies have shown that *Tribulus terrestris* contains a rich variety of phytochemical constituents including alkaloids, flavonoids, lignans, phenols, and terpenes. These compounds have been found to possess various biological activities and have shown potential for the management of hepatitis B, nephrolithiasis, and painful disorders. The significance of inhibiting kidney stones, or nephrolithiasis, is a common and painful condition characterized by the formation of small, hard deposits in the kidneys, which can cause severe pain and discomfort as they pass through the urinary tract. In addition to physical discomfort, kidney stones can also lead to complications, such as urinary tract infections, kidney damage, and obstruction of urine flow.

It is estimated that approximately 12% of the population will develop kidney stones at some point in their lives, making this a significant health concern. Therefore, prevention and treatment of kidney stones is a major focus of research in the field of urology. The current methods for the prevention and treatment of kidney stones include lifestyle modifications, dietary changes, pharmaceutical interventions, and surgical procedures. However, these approaches often have limitations such as potential side effects, high cost, and invasiveness. The extraction of phytochemicals from plants has gained significant attention in recent years, owing to their potential therapeutic benefits. *Tribulus terrestris* has been of particular interest in phytochemical extraction studies because of its medicinal properties and the presence of various bioactive compounds. *Tribulus terrestris* is commonly used in traditional Chinese medicine (TCM) to treat genitourinary disorders.

Phytochemicals are natural compounds found in plants that have been shown to possess various biological activities, including anti-inflammatory, antioxidant, and antihepatotoxic effects. In the context of kidney stones, phytochemicals extracted from plants may inhibit stone formation. Within the genus Tribulus, which comprises 25 species, many species are categorized as noxious weeds. The spiny characteristics of

its fruit pose a hazard to grazing animals. The roots and fruits of *Tribulus terrestris* possess a diverse array of medicinal properties. These include sweetness, cooling, diuretics, appetite, and digestive, anthelmintic, anti-inflammatory, alterative, laxative, cardiotonic, and tonic qualities. They have proven beneficial in addressing conditions such as strangury, dysuria, vata and pitta imbalances, renal and vesical calculi, anorexia, helminthiasis, spermatorrhoea, anemia, and general weakness. In contrast, the leaves exhibited diuretic, anthelmintic, and tonic characteristics. The diuretic properties of the plant are attributed to the presence of large quantities of nitrates and essential oils in the seeds. *Tribulus terrestris* is adaptable to both temperate and tropical climates and is recognized for its sharp thorns emanating from its seeds. While fruits are the most widely used parts, young stems, leaves, and roots have also found applications in herbal medicine.

Clinical trials have demonstrated its efficacy in reducing the number and size of kidney stones and alleviating associated symptoms, with a significant reduction in kidney stone size observed in a 90-day randomized open-label clinical trial (Rahman et al., 2017). Animal studies have also indicated its potential in reducing kidney stone formation and reversing early stage urolithiasis. In vitro research suggests that *Tribulus terrestris* not only inhibits the nucleation and growth of calcium oxalate crystals but also plays a cytoprotective role. Flavonoids present in this plant, such as kaempferol, astragalin, kaempferol-3-rhamnoglycoside, tribuluside, and rutin (Semerdjieva et al.,2019), contribute to its diuretic, antiurolithic, immunomodulatory, hypolipidemic, cardiotonic, hepatoprotective, anti-inflammatory, analgesic, antispasmodic, and antibacterial activities.

The high flavonoid content in *Tribulus terrestris* extracts is associated with anti-urolithiasis properties owing to its diuretic, antioxidant, anti-inflammatory, antibacterial, and preventive properties. In contrast to allopathic medicines, which primarily target specific aspects of urolithiatic pathophysiology, many plant-based therapies have demonstrated effectiveness at various stages of stone pathophysiology.

Existing extracts exhibit antilithogenic properties through diverse mechanisms, including promoting the spontaneous passage of calculi by increasing urine volume, pH, and anti-calcifying activity (diuretic activity). They also contribute to balancing the inhibitors and promoters of crystallization in urine, influencing crystal nucleation, aggregation, and growth (crystallization inhibition activity). Moreover, these plant-based therapies relieve the binding mucin of calculi (lithotriptic activity), improve renal function, regulate oxalate metabolism, address crystalloid colloid imbalance, enhance renal function to prevent urinary calculi recurrence, and improve the renal tissue antioxidant status and cell membrane integrity (antioxidant activity). Various herbal formulations, such as Cystone, Neeri, Uritone, Uriflow, Culdisol, Calcury, Chandraprabhabati, and Culin Forte, which contain *Tribulus terrestris*, are marketed globally to dissolve urinary calculi in the kidney and urinary bladder.

Tribulus terrestris, also known as "gokshura" in Indian Ayurveda practice, is a vital constituent in tonics and is featured in various antiurolithiatic herbal formulations. This project aimed to investigate the effect of *Tribulus terrestris* on kidney stones through comprehensive molecular docking and molecular dynamics studies. The primary objective of this study was to elucidate the molecular interactions between the bioactive compounds present in *Tribulus terrestris* and key targets associated with kidney stone formation. The project also sought to explore the antioxidant activity of *Tribulus terrestris* to understand its role in mitigating oxidative stress, a crucial factor in the

pathophysiology of kidney stones. The ultimate goal was to contribute to the scientific understanding of the molecular mechanisms underlying the efficacy of *Tribulus terrestris* in addressing kidney stones, which could have potential clinical applications. To do this soxhlet extraction, along with a qualitative phytochemical evaluation, antioxidant assay using DPPH, GCMS, Molecular docking, Molecular Dynamics and SASA analysis methods are used.
Soxhlet extraction is a time-tested method for the extraction of bioactive organic compounds from solid samples. In this technique, the sample was repeatedly extracted with a solvent and the extracted compounds were collected in a separate container. These compounds include saponins, alkaloids, and flavonoids, all of which contribute to the medicinal properties of *Tribulus terrestris*. Although Soxhlet extraction is time-consuming, the continuous extraction process ensures high yield. Researchers can subsequently analyze concentrated extracts using techniques such as gas chromatography (GC) or high-performance liquid chromatography (HPLC) to identify and quantify specific compounds.

In this technique, the sample is placed in a thimble in methanol, a polar solvent, because it is effective in extracting a wide range of phytoconstituents (Pallavi bhokare et al., 2018). The solvent continuously cycles through the sample, ensuring thorough extraction without pre-drying. Other solvents such as ethanol, hexane, and chloroform can also be used. The extraction time typically ranged from 2 h to 24 h. The Soxhlet method operates below the normal boiling point of the solvent, thereby enhancing solute solubility. The extractor contained the sample (in a thimble) and was connected to the condenser.

The condenser cooled the solvent vapors, causing them to condense and drip into the sample chamber. Solvent Reservoir Flask holds the solvent. The heating mantle provides controlled heating to vaporize the solvent. The extracted solution was collected from the collection flask after cycling through the sample. Methanol is widely used in Soxhlet extraction. The polarity of methanol allows it to dissolve a wide range of compounds, including lipids, alkaloids, and flavonoids, present in plants, such as *Tribulus terrestris*. Methanol can effectively extract polar and nonpolar compounds, which makes it suitable for diverse applications. Compared to other solvents, methanol is relatively safe to handle and has low toxicity.

Phytochemical analysis and antioxidant assays are critical for evaluating plant-derived compounds for their potential health benefits. Phytochemicals, bioactive compounds found in plants, have garnered significant attention owing to their diverse biological activities, including antioxidant, anti-inflammatory, and anticancer properties. These compounds are crucial for plant defence mechanisms and have shown promise for various therapeutic applications. Qualitative phytochemical analysis involves the identification and characterization of the phytochemicals present in plant extracts.

This analysis helps to determine the presence of various classes of compounds, such as alkaloids, flavonoids, terpenoids, phenolics, and saponins. Each class of phytochemical possesses unique chemical structures and biological activities that contribute to the overall medicinal properties of plant extracts.

Antioxidant assays are essential to assess the ability of plant extracts to scavenge free radicals and mitigate oxidative stress. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a widely used method to evaluate the antioxidant capacity of plant extracts. This

assay measures the ability of antioxidants to neutralize DPPH radicals, providing valuable information on the radical scavenging potential of the phytochemicals present in the extract. In the context of *Tribulus terrestris* and its potential impact on inhibiting kidney stones, qualitative phytochemical analysis will help to identify the specific phytochemicals present in the plant extract obtained through Soxhlet extraction using methanol as a solvent. The antioxidant assay with DPPH assesses the antioxidant activity of these phytochemicals, shedding light on their potential role in combating oxidative stress associated with kidney stone formation.

Furthermore, integrating advanced techniques, such as Gas Chromatography-Mass Spectrometry (GC-MS), molecular docking, and molecular dynamics simulations, will provide a comprehensive understanding of the molecular interactions between phytochemicals from *Tribulus terrestris* and key biomolecules involved in kidney stone formation. By combining traditional extraction methods with modern analytical tools, this study aimed to elucidate the therapeutic potential of phytochemicals from *Tribulus terrestris* in preventing kidney stone formation through their antioxidant and bioactive properties.

1. OBJECTIVES

- To conduct Soxhlet extraction and perform phytochemical analysis of *Tribulus terrestris* using methanol as a solvent to obtain the plant extract.
- Evaluate the antioxidant properties of the extract through DPPH assay to assess its potential in reducing oxidative stress associated with kidney stones.
- Utilize GCMS, molecular docking, and molecular dynamics techniques to understand the interaction between identified compounds and key components involved in kidney stone formation.
- Analyze and interpret the results to establish a correlation between the identified compounds and their therapeutic effects on kidney stones.

REVIEW OF LITERATURE

The pathogenesis of calcium oxalate stone formation is a multistep process and essentially includes nucleation, crystal growth, crystal aggregation, and crystal retention. Various substances in the body have an effect on one or more of the above stone-forming processes, thereby influencing a person's ability to promote or prevent stone formation. Promoters facilitate the stone formation while inhibitors prevent it. Besides low urine volume and low urine pH, high calcium, sodium, oxalate and urate are also known to promote calcium oxalate stone formation. (Aggarwal et al., 2013)

Calculi that consist predominantly of calcium phosphate occur more often in women than in men. Reports indicate a higher rate of recurrence in stones with a greater fraction of calcium phosphate (Tiselius, 1992).

The aim of the study is a comprehensive and critical assessment of the scientific publications involving TT, with special reference to its chemical constituents and biological properties that may facilitate current understanding and future studies of this fascinating plant species. (Semeredjiva et al., 2019)

This research explores the qualitative and quantitative phytoconstituents in hydroalcoholic and ethyl acetate extracts of fruits from *Tribulus terrestris* and *Solanum nigrum*. Additionally, the study seeks to assess the antioxidant activity of these extracts through various assays. The focus is on identifying and quantifying specific phytoconstituents like tannins, saponins, flavonoids, and terpenoids in the fruits of both plants, aiming to contribute to the understanding of the antioxidant potential and health-related benefits of these traditional medicinal plants. (Goel et al., 2022)

The study aims to assess the anti-urolithiatic activity of selected medicinal plants (Kalanchoe pinnata, Acalypha indica, *Tribulus terrestris*, Aerva lanata, and Boerhaavia diffusa) through in vitro, in vivo, and insilico methods. In vitro experiments focus on calcium oxalate crystallization, while in vivo evaluation involves a polyherbal formulation administered to rats with induced urolithiasis. Insilico studies include molecular docking of plant constituents with relevant protein targets. The overall goal is to comprehensively understand the anti- urolithiatic potential of these medicinal plants. (Swetha et al., 2022)

This study was aimed at evaluating the anti-hyperuricemic potentials of a flavonoid aglycone-rich fraction of T. terrestris leaf extract in vivo in mice and to provide structure- based molecular frameworks for its XOD inhibitory action mechanism via molecular docking and molecular dynamics simulations, validating all XOD-flavonoid interactions by an in vitro enzyme activity assay with the aid of commercially available Bovine XOD and pure isorhamnetin, quercetin and kaempferol with an ultimate view to providing functional and molecular platforms for the possible discovery of flavonoid- based uricostatic anti-hyperuricemic drug leads. (Ajala et al., 2022)

The study aims to identify and characterize compounds isolated from *Tribulus terrestris*, including two oligosaccharides and a stereoisomer of di-p-coumaroylquinic acid. The structures were determined using various spectroscopic methods, and notably, the NMR spectral data for a known compound (4,5-di-p-trans-coumaroylquinic acid) is reported for the first time. The investigation further explores the antioxidant activity of di-p-coumaroylquinic acid derivatives, highlighting their significant contribution to the plant's overall antioxidant effect. (Hala M.Hammoda et al., 2013)

This clinical trial investigates the efficacy of Safoof-e-Khar-e- Khasak (*Tribulus terrestris*), a Unani medicine, in managing Hisat-ul-Kuliyah (Nephrolithiasis). Through statistical analysis, the study assesses the medicine's impact on pain relief, diuresis, and reduction in stone size, emphasizing its significance in treating the disorder. (Md. Najibur Rahman et al., 2017)

This investigation aims to assess the impact of aqueous extracts from *Tribulus Terrestris* fruits and Urtica dioica leaves on sodium oxalate-induced renal calculi in male albino rats. The study evaluates the extracts' effects on biochemical markers, antioxidant enzyme activity, and histopathological changes, with a focus on understanding their potential in mitigating kidney stone formation and associated complications. (Soha S Mohamed et al., 2023)

Gas chromatography mass spectrometry (GC/MS) analysis is an effective testing and troubleshooting tool to identify and quantify chemicals in a complex mixture and

GCMS analysis are used in various fields. GC and MS provide distinct but complementary results; while GC separates components of a mixture, MS can analyse and identify these components. These methods were first used in tandem in the 1950s, and are still widely applied in clinics and laboratories worldwide. (Adam Nugraha et al., 2021)

This study provides an overview of molecular docking, focusing on computational modeling of molecular complexes. It discusses the prediction of three-dimensional structures based on ligand-target binding properties. The study highlights different types of molecular docking approaches, such as flexible ligand docking and rigid body docking. Additionally, it emphasizes the applications of molecular docking in lead optimization and rational drug design. (Shweta Agarwal et al., 2016)

A comprehensive evaluation of pharmacognostic properties, such as Fluorescence analysis, Weight loss upon drying, Moisture content, Total ash, Acid insoluble ash, Water soluble ash, Ignition residue, Extractive values, Initial phytochemical analysis, and Thin layer chromatographic studies, was carried out on various extracts of the plant *Tribulus terrestris*. The preliminary phytochemical analysis of the crude drugs revealed the presence of saponins, reducing sugars, triterpenoids, steroids, tannins, and alkaloids in the sample extracts. Notably, the pet. Ether and chloroform extract of *Tribulus terrestris* were found to contain flavonoids. (Neha singh et al., 2022)

In this research, the antioxidant activity was assessed using the DPPH (1,1 diphenyl-2-picrylhydrazyl) radical scavenging technique for the methanolic extraction fractionation of the entire *Tribulus terrestris* plant, sequentially with toluene and n-butanol. The fractions obtained were condensed under diminished pressure to produce the corresponding antioxidant activity. The methanolic extract of a butanol fraction from *Tribulus terrestris* demonstrated superior antioxidant capacity, as measured by the DPPH radical scavenging method, when compared to the standard, ascorbic acid. (Srisailam K et al., 2017)

The research indicates that the methanolic extract of *Tribulus terrestris* exhibits considerable antioxidant properties in vitro. Among the chosen phytochemicals for the In-silico study, Heptacosane, apiol, and palmitic acid demonstrated effective interactions with the target protein. Notably, Heptacosane emerged as a promising candidate for the development of a new lung cancer drug. The findings suggest that T. terrestris, a significant medicinal plant, is a powerful source of natural antioxidants and has potential anticancer properties, supporting its conventional use in eco-friendly therapeutics. Further investigations on *Tribulus terrestris* could contribute to drug discovery for the betterment of human health. (Siddique et al., 2022)

Glycolate oxidase (GOX) plays a key role in the oxalate synthesis pathway, catalyzing the conversion of glycolate to glyoxylate, which is subsequently transformed into oxalate. The therapeutic targeting of GOX has shown promise in addressing calcium oxalate urolithiasis. This study sought to identify potential GOX inhibitors from traditional sources. Molecular modeling of the identified leads, namely quercetin and kaempherol, was conducted using Glide 5.5.211 (SchrodingerTM suite). Due to the unavailability of pure human glycolate oxidase (hGOX), in vitro tests were carried out on spinach glycolate oxidase (sGOX), which shares 57% identity and 76% similarity with hGOX, including several common conserved active site residues. The goal of the study was to uncover a potential mode of action for the anti-GOX leads from *Tribulus*

terrestris, contributing to the development of anti-urolithic drugs. The findings suggest potential for the development of future GOX inhibitory leads. (Shrifule AL et al., 2011)

This computational study shows the selection of phytochemicals, including catechin, epicatechin, gallic acid, gallocatechin, epigallocatechin, epigallocatechin 3-o-gallate, 4-methoxy-nor-securine, nor-securinine, and fisetin, which were examined for their interactions with human glycolate oxidase (hGOX) and oxalate oxidase (OxO). Among these, gallic acid, gallocatechin, and fisetin demonstrated superior docking scores.The molecular dynamic (MD) simulation analysis revealed consistent interactions of gallic acid with both hGOX and OxO, and of gallocatechin and fisetin with hGOX. Specifically, gallic acid formed stable bonds with TYR26, LYS 236, ARG 315, and ASP 291 residues of hGOX, and with GLU 58 residue of OxO. Gallocatechin established stable bonds with TYR 26, ASP 170, ARG 167, and THR 161 residues of hGOX. Fisetin, as predicted in the molecular docking study, formed stable bonds with TYR 26, TRP110, and ARG 263 of hGOX during the MD simulations. However, no interactions were observed during the MD simulation of OxO with gallocatechin and fisetin. Collectively, these findings indicate that gallic acid, gallocatechin, and fisetin could be promising candidates for the development of plant-based treatments for kidney stones in humans. (Nageshwari P et al., 2023)

MATERIALS AND METHODS

Materials required: Plant samples, Grinder Scissors, Soxhlet extraction apparatus, Round bottom flask, Conical flask, Funnel, Distilled water, Condensation apparatus, Test tubes, Pipettes and tips, Capillary tube, Beakers, Eppendorf tubes, Methanol, DPPH (2,2-diphenyl-1-picrylhydrazyl) 0.02 M potassium ferrocyanide,
0.01 M ferric chloride FeCl2, aqueous hydrochloric acid (HCl), distilled water, olive oil, dilute ammonia, concentrated sulfuric acid, chloroform, glacial acetic acid, Wagner's reagent, and sodium hydroxide.

Plant Material: The plant material (leaves, roots, stems) were collected from Erode district. Collected *Tribulus terrestris* plant and shade dried them to remove moisture. Cleaned and ground the plants into a fine powder using an electric mixer. Ensured it is thoroughly dried to enhance extraction efficiency before grinding.

Preparation of extract: The Soxhlet extraction method is extensively employed to extract bioactive compounds from diverse natural sources. Weighed 15g dried and powdered *Tribulus terrestris* material. The weighed plant material is placed in the thimble of the soxhlet extraction apparatus. Added adequate volume of methanol (solvent – 250ml) to the round-bottom flask of the Soxhlet apparatus. Set up the Soxhlet apparatus and started the extraction process. The solvent vaporizes, rises to the condenser, and then drips onto the plant material in a cyclic process. As the solvent reaches its overflow point, a siphon draws up the solution from the thimble-holder, returning it to the round bottom flask along with the extracted solutes. The solutes are retained in the flask, while the solvent goes back to the sample bed. This process was done for 3 hours at a temperature of 60 degree celcius.

Phytochemical analysis:

By conducting screenings for phytochemicals, we can uncover the constituents within plant extracts and pinpoint the most prevalent ones.

Steps:

10 test tubes are taken and labelled with the names of phytochemical compounds. The extracted sample is diluted using methanol. The test tubes are kept in test tube stand for adding respective reagents for the phytochemical study.

1. Test for Tannin: Take 1ml of plant extract and mix it with 1ml of potassium ferrocyanide with a molarity of 0.02 M, and 1ml of ferric chloride FeCl2 with a molarity of 0.01 M. The presence of tannin is indicated by a blue-black coloration.
2. Test for Phlobatannin: Boil 1ml of plant extract with aqueous HCl; the presence of a red precipitation indicates the presence of phlobatannin.
3. Test for Saponin: Mix 1ml of plant extract with distilled water and add a few drops of olive oil; the formation of foam indicates the presence of saponin.
4. Test for Flavonoids: Combine 1ml of plant extract with 5ml of dilute ammonia and add a few drops of concentrated sulfuric acid; the formation of a yellow colour indicates the presence of flavonoids.
5. Test for Steroids: Mix a small amount of plant extract with 2ml of chloroform in the presence of concentrated sulfuric acid (H2SO4); the formation of a red colour indicates the presence of steroids.
6. Test for Alkaloids: Add a few drops of Wagner's reagent to 1ml of plant extract; the appearance of a reddish-brown precipitate indicates the presence of alkaloids.
7. Test for Quinones: Combine 1ml of plant extract with dilute sodium hydroxide; the appearance of a blue-green or red colour indicates the presence of quinones.
8. Test for Coumarin: Mix 1ml of plant extract with 10% sodium hydroxide in the presence of chloroform; the appearance of a yellow colour indicates the presence of coumarin.
9. Test for Terpenoids: Combine 1ml of plant extract with 2ml of chloroform in the presence of 3ml of concentrated sulfuric acid; the appearance of a reddish-brown colour indicates the presence of terpenoids.
10. Test for Cardiac Glycosides: Add 2ml of glacial acetic acid and 1 drop of ferric chloride to a sample of plant extract in the presence of 1ml of concentrated sulfuric acid; the formation of a brown ring, violet ring, or thin layer of greenish ring indicates the presence of cardiac glycosides

Antioxidant assay:

Antioxidant assays are crucial tools used in the evaluation of substances' ability to combat oxidative stress, a process linked to aging and various diseases. These methods assess different aspects of antioxidant activity, helping researchers quantify and compare the antioxidant potential of compounds. Chemical antioxidant assays include the determination of scavenging ability against free radicals such as DPPH, superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, and nitric oxide.

DPPH ASSAY: DPPH, or 1,1-diphenyl-2-picrylhydrazyl, is a stable free radical known for its deep violet colour. When an antioxidant, a substance capable of donating a hydrogen atom, is introduced to the DPPH solution, it causes the DPPH's unpaired electron to pair up, transforming the DPPH into 1,1-diphenyl-2-picrylhydrazine. This transformation triggers a shift in colour from violet to yellow. The extent of this colour shift serves as an indicator of the antioxidant's ability to neutralize free radicals, which is present in the plant extract. This is a key measure of the antioxidant's potential to scavenge free radicals.

1ml of 0.4mM solution of DPPH was added to 3ml of different concentrations of extract sample – 100, 200, 400, 600, 800, 1000 µL and allowed for incubation at room temperature for 30 minutes in a dark room. After 30 minutes, the absorbance was read at 517 nm against blank samples containing methanol using a double beam spectrophotometer. The total %DPPH radical scavenging is calculated by using the standard equation mentioned below:

Radical scavenging activity/inhibition(%)=(Abs control–Abs sample/Abs control)x100

GCMS:

The sample was investigated through Gas Chromatography Mass Spectrometry/Mass Spectrometry (GC-MS) or Gas chromatography with single quad mode. The GC-MS is Agilent 5977 MSD model. The GCMS- Agilent 5977 MSD is a single quadrupole gas chromatograph-mass spectrometer with fused silica capillary column HP-5MS (5%-phenyl)-methylpolysiloxane and Length: 30m; Internal diameter: 0.25 mm; Thickness: 0.25 µm. carrier helium gas was used at constant flow rate 1ml/min and injection volume of one microliter was used in split ratio. The column flow rate was 1.2ml/min with a purge volume of 3ml/min. The injector temperature was 230°C with split injection mode. The oven temperature was programmed from 75°C, with an increase of 5°C/min, ending with a 5 min isothermal at 300°C and total GC running time was 53.5 minutes. The oven temperature was programmed from 50°C, with an increase of 5°C/min, ending with a 5 min isothermal at 350°C. A scan interval of 3 seconds and fragments from 50 to 600 m/z was programmed with a scan speed of 1562 amu/sec.

Molecular Docking:

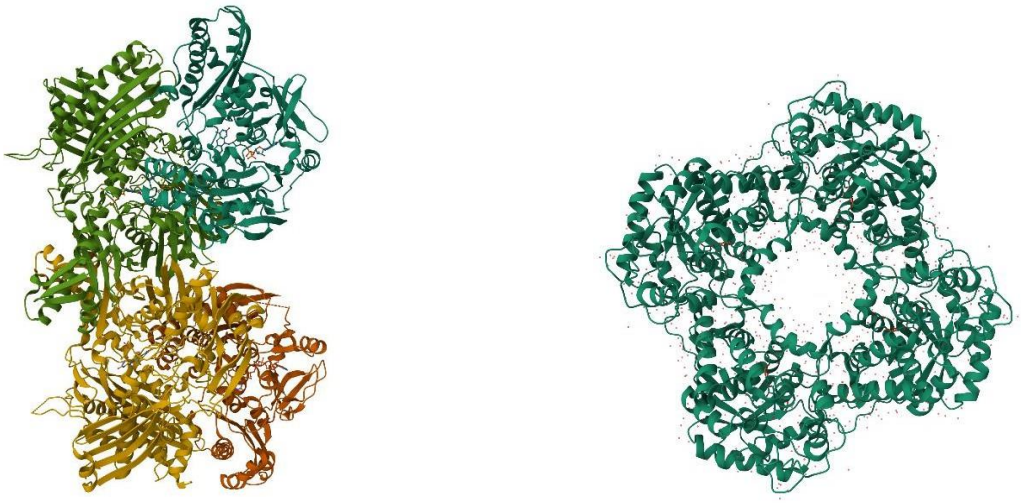
The structures of the proteins and ligands were obtained from the RCSB Protein Data Bank and PubChem. The proteins and ligands were first pre-processed using PyMOL and Avogadro. In PyMOL, the water molecules in chains B, C, and D were removed to simplify the interaction between the protein and ligand. Avogadro was employed to add hydrogen atoms to the ligand to refine its structure. Precise addition of hydrogen atoms to the protein structure ensures an electrostatically neutral environment. This step enhances the accuracy of the subsequent calculations. Additionally, we carefully and precisely assigned Kollman charges to the protein atoms to create an accurate representation of their electronic structures.

The evaluation of atomic contacts and non-bonded interaction energies relies on the assignment of atomic radii, which is based on the Adams and Defrees (AD4) parameters. Ligand preparation is critical. This involves identifying the ligand's root atom and fine-tuning the torsion tree parameters. This optimization allowed us to effectively explore the potential binding modes.

We utilized the binding site coordinates (X,Y,Z) from Discovery Studio (For 1JRP, 0.031302, 43.396868, and 0.616094. For 2RDU, 27.020839, -9.589968, -15.347548).

Subsequently, we employed the Autogrid module in AutoDock 4 to generate a grid representation of the protein-ligand interaction space. The successful creation of a grid log file (glg) confirms this step. Before launching AutoDock, we configured critical parameters, including setting the number of genetic algorithms to 100. The chosen configuration results in an input file in the Lamarckian Genetic Algorithm (LGA) format, a method known to efficiently explore ligand conformational space and predict potential binding modes. Upon executing AutoDock, we obtained a comprehensive output file in the dlg format containing a spectrum of binding poses. Our subsequent analysis identified the most promising protein-ligand interaction pose.

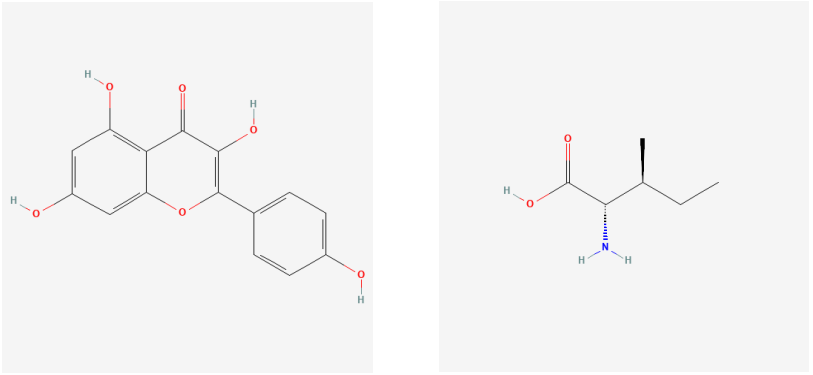
The five ligands, Kaempferol, L-isoleucine, Phytol, Neophytadiene, and Squalene were pre-processed along with both the proteins 1JRP and 2RDU using above steps. All the compounds were selected from the GCMS result, except kaempferol, it was selected by referring to the research papers due to its good binding interactions with the target proteins. To identify the best pose, each ligand was docked with both the proteins individually. A total of 10 docking outputs were obtained.



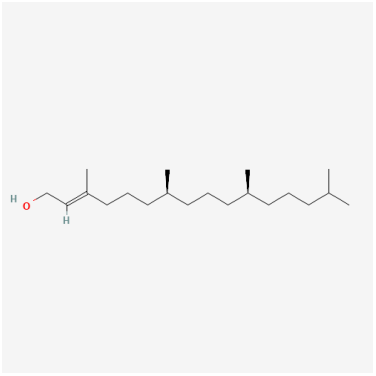
(A) (B)

Figure 1: shows the 3D image of protein (A) - 1JRP and (B) - 2RDU

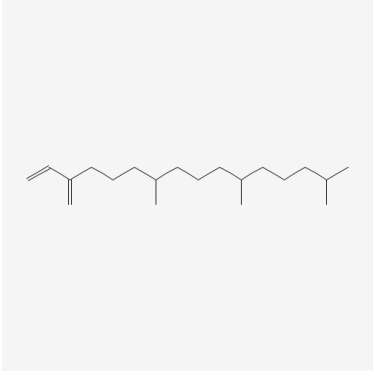
These two proteins are involved in the process of formation of kidney stones (urolith). (Swetha G S et al., 2022). Protein 2RDU is Human Glycolate Oxidase in Complex with Glyoxylate and protein 1JRP is Xanthine Dehydrogenase inhibited by alloxanthine from Rhodobacter capsulatus.



Kaempferol

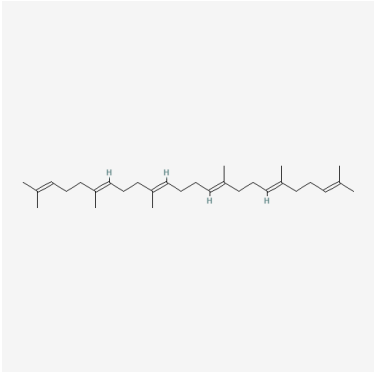


L-Isoleucine



Phytol

Neophytadiene



Squalene

Figure 2: shows the 2D images of the ligands used for molecular docking studies.

Molecular Dynamics:

From the output obtained by molecular docking, the best compound with high binding affinity was taken for the MD simulation to analyse the stability of the protein-ligand complex. The protein – ligand complex of "Phytol with 2RDU was subjected for the molecular dynamics simulation from the above output obtained from molecular docking. The process of molecular dynamics simulation was set up using the Solution Builder from CHARMM-GUI, which made it easy to create the input file. The best shape of the protein-ligand complex was first prepared and loaded as a PDB file. In particular, Chain A of 2RDU protein was chosen along with the heteroatom (UNL). The ligand was characterized using the CHARMM General Force Field with a pH of 7.

After characterizing the ligand, basic ions, such as KCL were added to the system using the Monte Carlo method. The protein was then placed in a rectangular waterbox and grid data for the PME FFT were automatically created to set up periodic boundary conditions. The equilibration input was created under the NVT ensemble, whereas the dynamics input was created under the NPT ensemble, with the temperature set to 303.15 K. The CHARMM 36 force field was used to create the input files for GROMACS. The final input file was compressed into a .tgz format for ease of handling.

Before starting the molecular dynamics simulation, the dynamics duration in step 4 of the input file was changed to simulate the system for 100 ns. Finally, the input file was run to perform the molecular dynamics simulation using GROMACS, by running the appropriate commands.

RESULTS AND DISCUSSION:

Extraction:

After 3 hours of Soxhlet extraction process at 60°C, the solvent was removed and transferred to a beaker. The excess solvent was removed by giving hot water bath for 2 hours at 50°C. The concentrated plant extract was transferred from the beaker to Eppendorf tubes using micro spatula.

Figure 3: Collected plant material *Tribulus terrestris.*

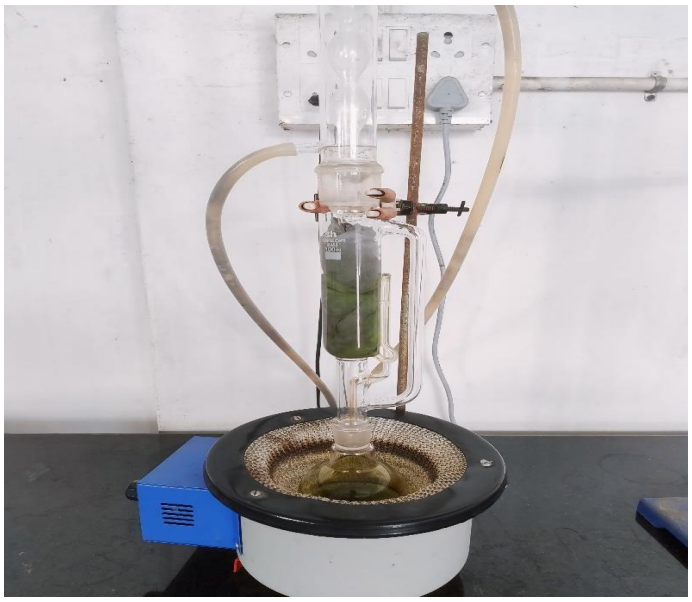


Figure 4: shows the soxhlet apparatus containing 15g of dried and powdered plant inside the thimble, with methanol as solvent in the flask.

Phytochemical analysis result:

It was found that all the below mentioned phytochemicals were present in moderate concentration in the plant extraction.

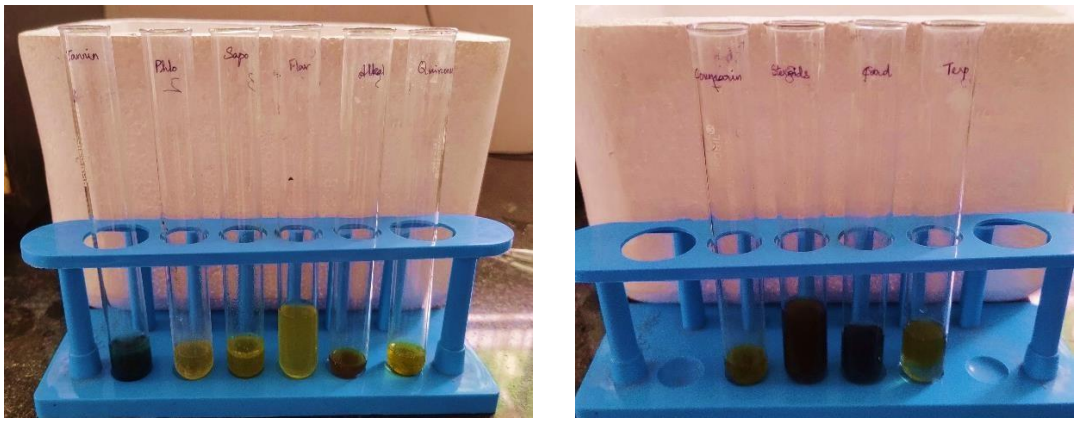


Figure 5: Phytochemical analysis.

S.NO	NAME OF TEST	RESULT
1	Test for Tannin	+
2	Test for Phlobatannin	+
3	Test for Saponin	+
4	Test for Flavinoids	+
5	Test for Steroids	+
6	Test for Alkaloids	+
7	Test for Quinones	+
8	Test for Coumarin	+
9	Test for Terpenoids	+
10	Test for Cardiac Glycosides	+

Table 1: Presence of phytochemicals.

Antioxidant Assay using DPPH:

It is one of the reliable, easy and precise method for evaluation of ability of antioxidants to scavange free radicals responsible for oxidative stress, and it is also useful in quantification of antioxidants. At 10µg/ml, DPPH scavenging activity of methanolic extract of *Tribulus terrestris plant* was found to be 92.13% on an average taken from various concentrations. The value of absorbance of control is 0.108.

Concentration of extract	**Absorbance**	**Percentage of scavenging**
100	0.120	88.88
200	0.113	95.37
400	0.122	87.03
600	0.119	89.84
800	0.113	95.37
1000	0.112	96.29

Table 2: shows the absorbance at 517nm and its %Radical scavenging activity.

GCMS Result:

The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. It indicated presence of many bioactive compounds out of which only few were in high concentration. They are shown in the table below.

Compound name	**Retention time (mins)**	**Area %**
L-Isoleucine	13.124	2.58
3-O-Methyl-d-glucose	18.514	68.04
Neophytadiene	22.594	3.51
Phytol	29.627	7.63
Squalene	41.871	8.88

Table 3: GCMS result – shows the characterized compounds.

Molecular Docking:

The molecular docking simulations were performed to investigate the potential binding modes and affinities of the ligands towards the target protein. The docking scores, which are indicative of the binding affinities, ranged from -9.04 Kcal/mol to -4.97 Kcal/mol. Molecular docking results of different chemical constituents of *Tribulus terrestris* with proteins 1JRP and 2RDU are compared in the Table 4. The best docking score was found to be -9.04 Kcal/mol for the ligand Squalene with protein 1JRP and -8.74 Kcal/mol for squalene with protein 2RDU. The molecular docking study provided valuable insights into the binding modes and affinities of the ligands towards the target protein.

Phytochemical constituents	Binding energy for the protein 1JRP (Kcal/mol)	Binding energy for the protein 2RDU (Kcal/mol)
Kaempferol	-6.83	-7.01
L- Isoleucine	-5.55	-4.97
Phytol	-7.64	-8.23
Neophytadiene	-7.09	-7.63
Squalene	-9.04	-8.74

Table 4: shows the least binding energy of the compounds in molecular docking.

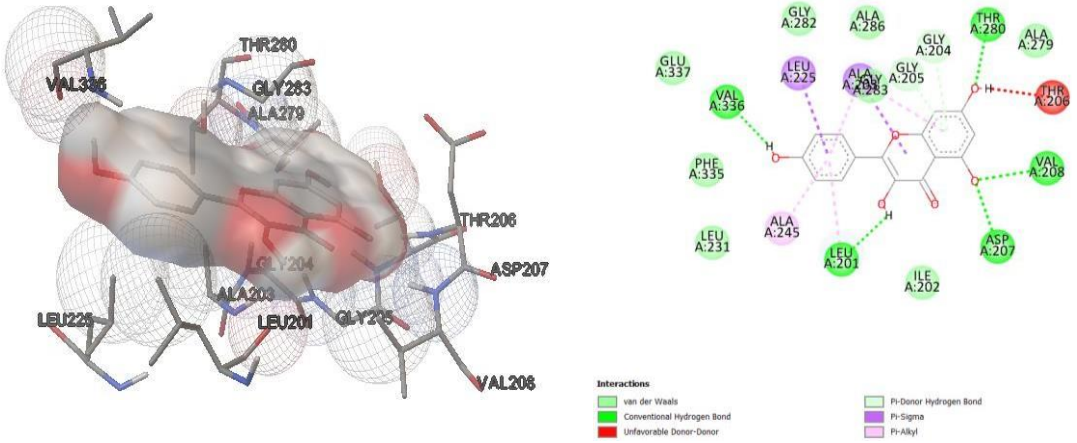


Figure 6: 3D and 2D ligand interactions of Kaempferol with protein 1JRP

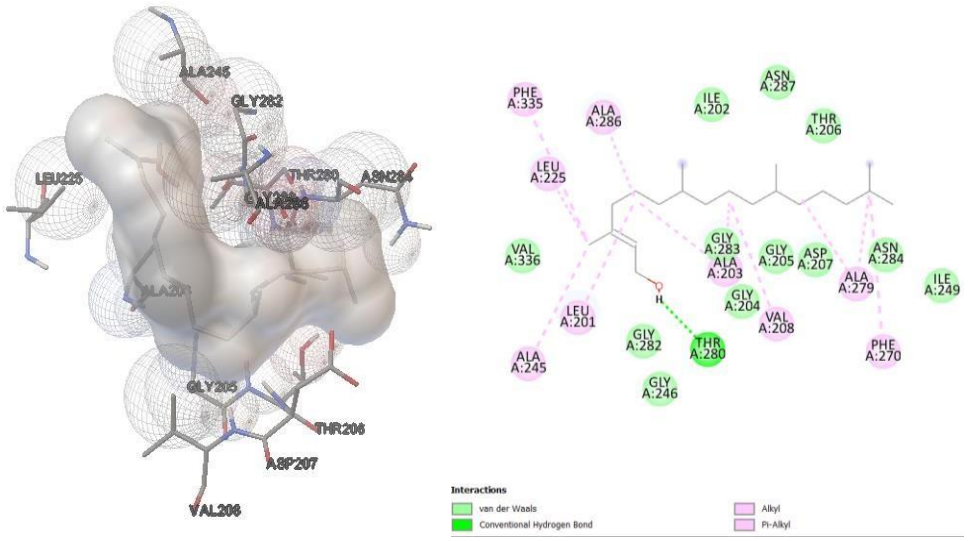


Figure 7: 3D and 2D protein-ligand interactions of 1JRP-Phytol.

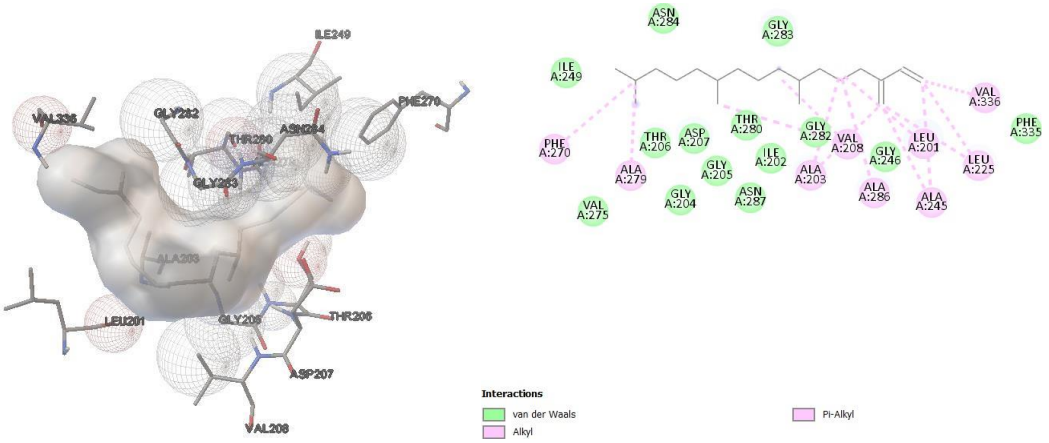


Figure 8: 3D and 2D protein-ligand interactions of 1JRP- neophytadiene.

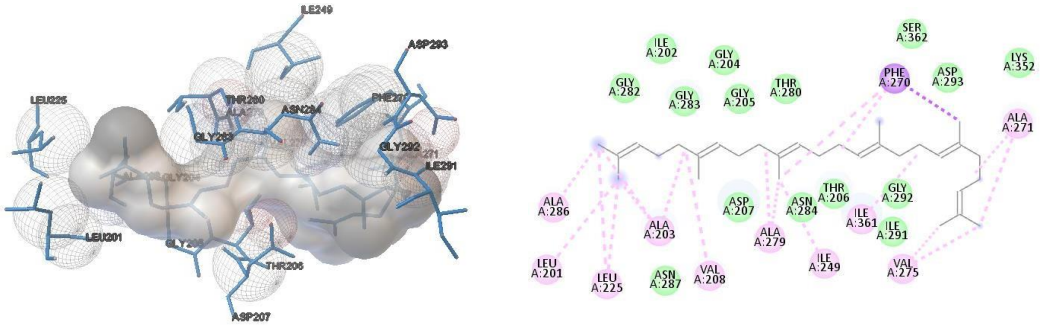


Figure 9: 3D and 2D protein-ligand interactions of 1JRP-Squalene.

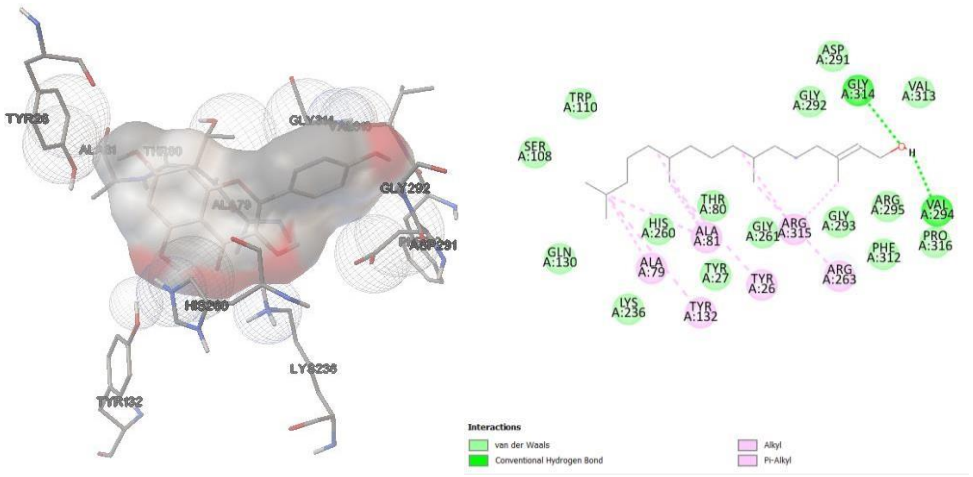


Figure 10: 3D and 2D protein-ligand interactions of 2RDU-Kaempferol.

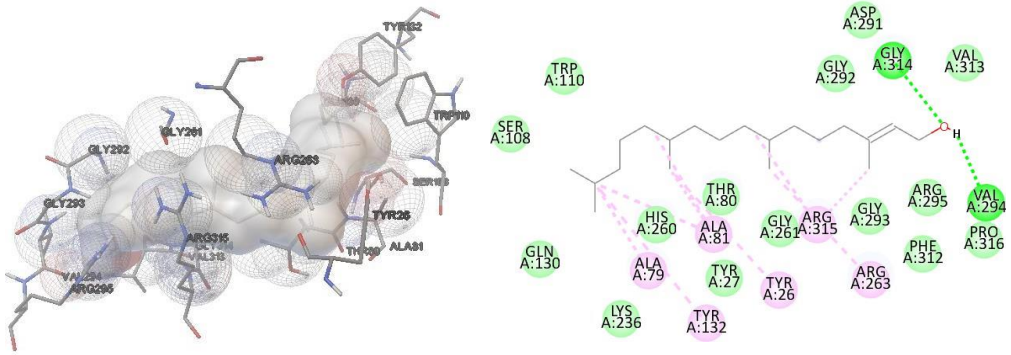


Figure 11: 3D and 2D protein-ligand interactions of 2RDU-Phytol.

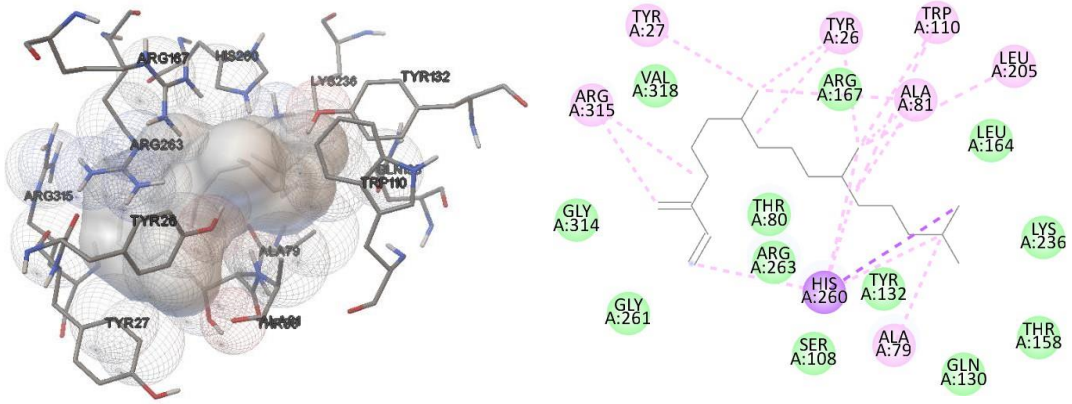


Figure12: 3D and 2D Protein-ligand interactions of 2RDU-Neophytadiene.

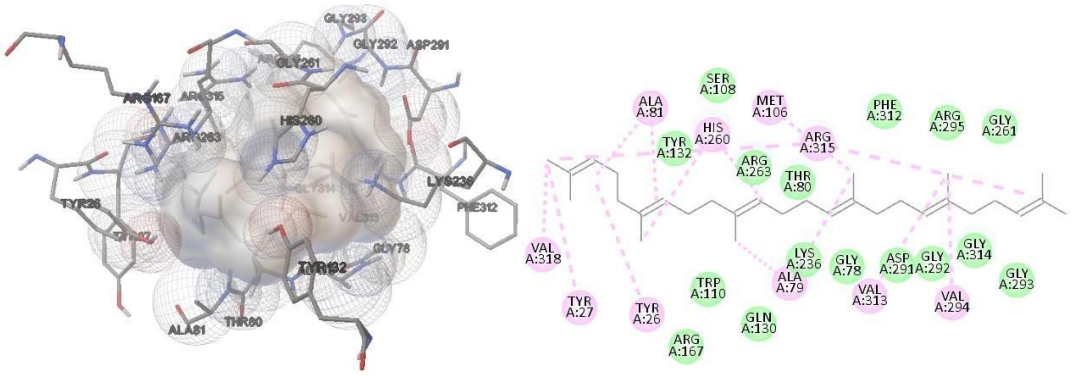


Figure 13: 3D and 2D Protein-ligand interactions of 2RDU-Squalene.

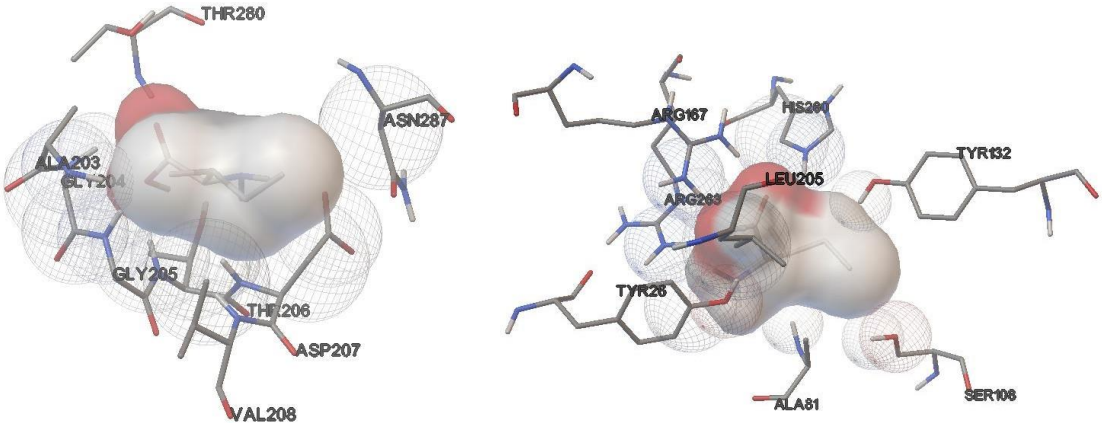


Figure 14: shows the 3D protein-ligand interactions of L-isoleucine with 1JRP and 2RDU respectively.

MOLECULAR DYNAMICS:

The next step involved subjecting the selected hit compound (phytol with protein 2RDU) to a 100 ns molecular dynamics (MD) simulation. This simulation aimed to assess the stability of the protein-ligand complex. During this process, we explored the binding mechanism and observed the dynamic behaviour of the complexes. Additionally, we analysed the conformational stability of the receptor-ligand interaction using metrics such as RMSD (Root Mean Square Deviation), RMSF (Root Mean Square Fluctuation), and the Radius of Gyration. We checked the changes in the secondary structure when selected compounds were bound to the protein.

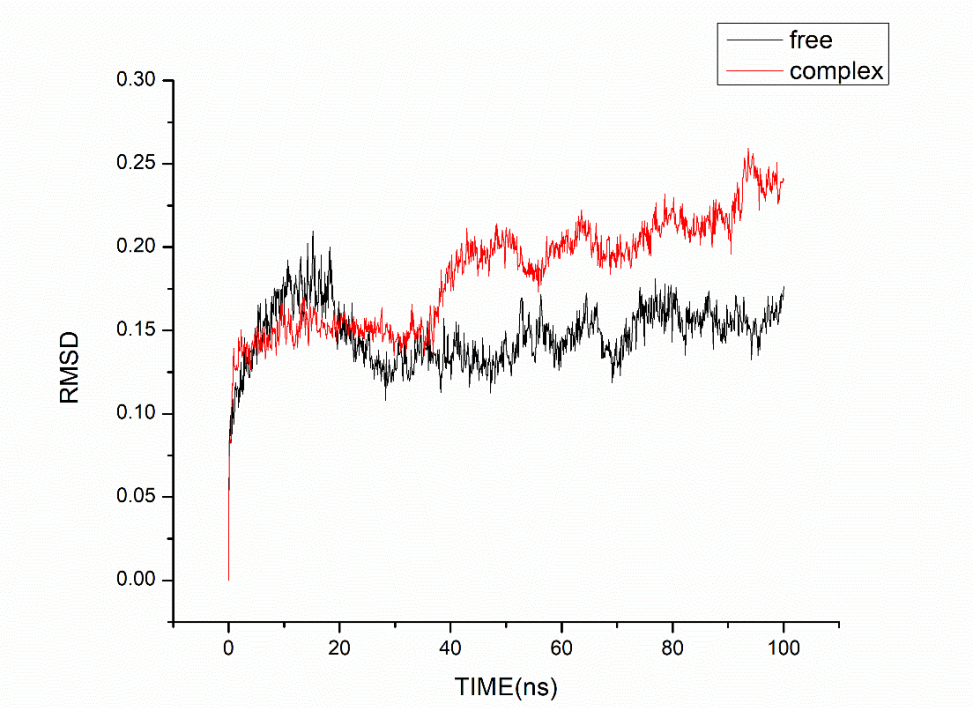


Figure 15: shows the RMSD plot for the interaction of protein with ligand which forms a complex.

During the molecular dynamics (MD) simulation, we monitored the RMSD (Root Mean Square Deviation) value to assess changes in the position within the ligand-receptor complex. Notably, there was an initial increase in the RMSD value during the early stages of the simulation up to 11 nm was observed for the complex. Then it increases steadily up to 21nm until 40ns of run time. From there it fluctuates and reaches a maximum of 25nm at the end. The “complex” line shows fluctuations, suggesting that the protein-ligand complex also deviates from its initial structure, but not as steadily as the free protein.

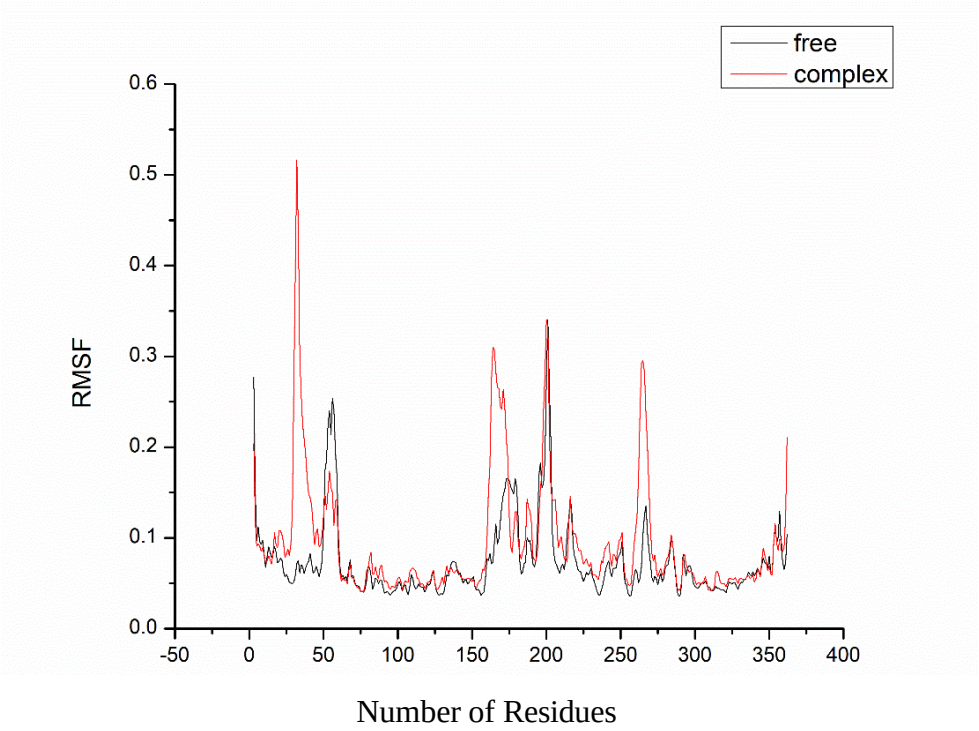


Figure 16: shows the RMS fluctuations plot for the interaction of protein with ligand which forms a complex.

Further, we checked the momentum of each residue throughout the simulation when the ligand binds to the protein through RMSF plot which was plotted against number of residues (RMSF VS Residues). The graph shows the RMSF values for both the free protein and the protein-ligand complex. Both lines show significant fluctuations, indicating that there are regions in both the free protein and the protein-ligand complex that are highly flexible and move around their average positions. The initial residues shows a RMSF of 2nm and up to 28 residues it shows a stable RMSF. From 28-33 it shows sudden fluctuation and again at residues from 150 to 380 large number of fluctuations are seen. This increase and decrease in the fluctuation are possibly due to interaction between the hit compound and the protein helix, small β-sheets, and the loop region of the protein. The RMSF plots backbone shows more fluctuation in the loop and helix regions.

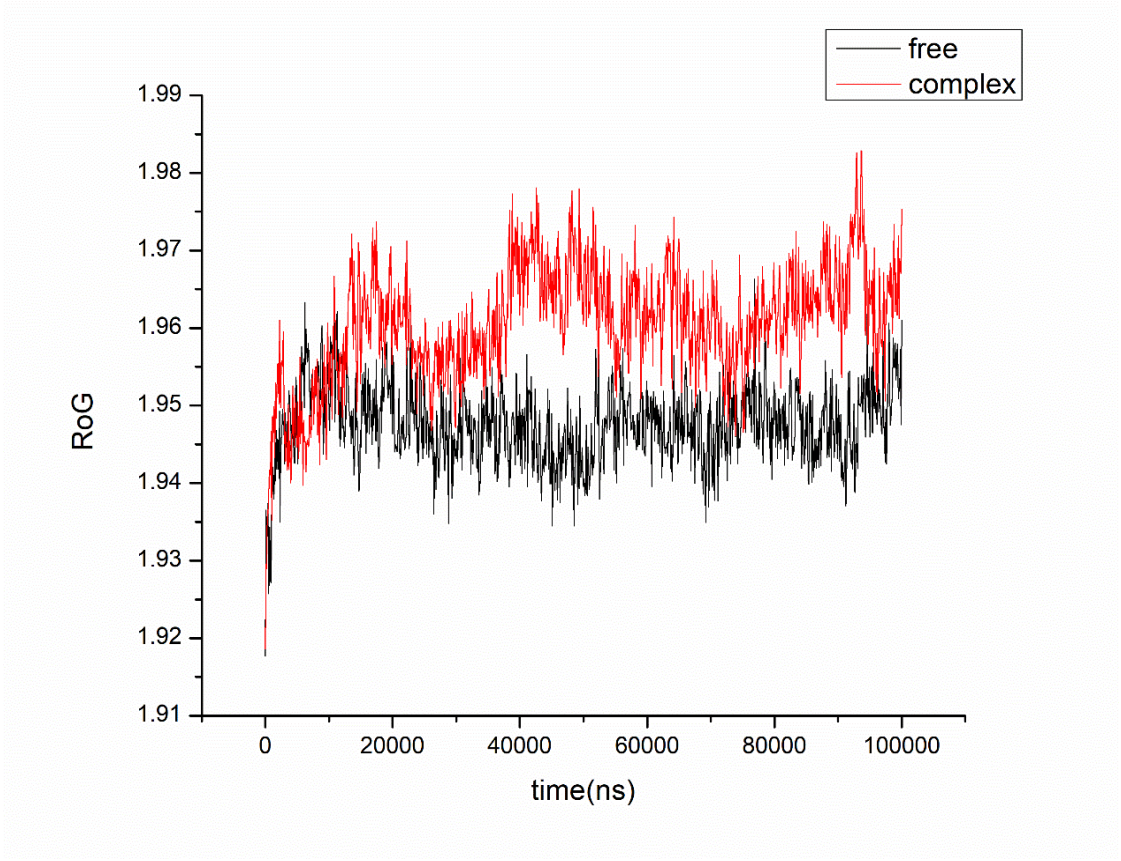


Figure 17: shows the Radius of gyration plot for the interaction of protein with ligand which forms a complex.

The above graph shows the Radius of Gyration for both the free protein and the protein-ligand complex. The Rg value lies between 1.94 to 1.98 nm with steady increasing pattern along with some significant fluctuations. A sudden decrease in Rg is seen at 25 ns where it falls from 1.97 to 1.94 nm. And again fluctuates to 1.97 nm at around 40 ns which is maintained steadily up to 100ns. Both lines fluctuate significantly and overlap each other at several points, suggesting that the overall shape and size of the protein and the protein-ligand complex change over time

In conclusion, these results suggest that the binding of the ligand affects the structural dynamics of the protein. It shows low RMSD, minimal fluctuations (low RMSF), and a consistent radius of gyration, it suggests that the protein-ligand complex is stable and maintains its binding conformation.

SUMMARY AND CONCLUSIONS:

Urolithiasis, classified as one of the most painful disorders afflicting mankind since ancient times, affects 2 - 20% population worldwide and is the third most common affliction of the urinary tract (Johri et al., 2010). Over the past two decades, significant advancements have been made in the management and prevention of kidney stones. This has been achieved through a combination of dietary modifications, surgical interventions, and medications. However, challenges such as side effects and recurrence persist. As a result, complementary approaches like phytotherapy are gaining traction.

Herbal medicines are increasingly being embraced due to the adverse effects and toxicity associated with conventional medicines. In both developed and developing

nations, plant-based medicines are highly sought after for primary healthcare. This is attributed to their broad spectrum of biological and medicinal properties, superior safety profile, and cost-effectiveness. Historically, a variety of treatments have been used to manage urinary stones. The current study focuses on exploring the potential of the methanolic extract of *Tribulus terrestris* in inhibiting calcium oxalate stones, using computational studies as a tool for investigation.

The project involved a detailed investigation into the bioactive compounds extracted from *Tribulus terrestris* and their potential impact on kidney stones. Through Soxhlet extraction using methanol, a diverse range of compounds including saponins, alkaloids, and flavonoids were obtained, known for their medicinal properties. Subsequent phytochemical analysis confirmed the presence of these compounds in the extract. Antioxidant assays demonstrated the extract's ability to scavenge free radicals, indicating its potential in reducing oxidative stress linked to kidney stones. GCMS analysis identified specific compounds within the extract, providing insights into its chemical composition.

Furthermore, molecular docking studies revealed interactions between the identified compounds and key proteins associated with kidney stone formation, such as Chain A of the 2RDU protein and the ligand. Molecular dynamics simulations further elucidated the dynamic behaviour of these compounds, offering insights into their potential effectiveness in treating kidney stones. The results collectively establish a correlation between the identified compounds and their therapeutic effects on kidney stones.

In conclusion, the study enhances the scientific understanding of how bioactive compounds from *Tribulus terrestris* could hold promise in addressing kidney stones. The research underscores the potential clinical implications of these compounds and emphasizes the need for continued exploration to leverage the therapeutic properties of natural products for managing kidney stones.

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