GMR
The Original

PHYTOCHEMICAL PROFILING AND IN SILICO EVALUATION OF SAPONIN-RICH EXTRACTS FROM PIPER NIGRUM ROOT FOR ANTIOXIDANT, ANTIDIABETIC AND ANTICANCER POTENTIAL AGAINST SKIN CANCER CELL LINES

Ramya Subramani[1], Kavitha Ramachandran[2], Sujatha Govindaraj[3], Shanthi Narayanan[4], Kaliya Perumal Reshma[5], Poornima Arumugam[6], Thiruselvi Muniswaran[7], Nirubama Kumar[8]* Rajendran Venkatesh[9]*

[1]Department of Biochemistry, Kongunadu Arts and Science College, Coimbatore - 641029, Tamil Nadu, India.
[2] Department of Botany, Holy Cross College (Autonomous), Affiliated to Bharathidasan University, Tiruchirappalli – 620002, Tamil Nadu, India.
[3] Department of Botany, Thanthai Periyar Government Arts and Science College (Autonomous), Tiruchirappalli - 620 023. Tamil Nadu, India.
[4] Department of Organon of Medicine, Vinayaka Mission's Homoeopathic Medical College and Hospital, Salem-636308, Tamil Nadu, India.
[5]Department of Botany, A.V.V.M. Sri Pushpam College (Autonomous), Poondi, Thanjavur, 613503, Tamil Nadu, India.
[6]Department of Biochemistry, Kongunadu Arts and Science College, Coimbatore - 641029, Tamil Nadu, India.
[7]Department of Biochemistry, Dr. N.G.P. Arts and Science College, Coimbatore - 641048, Tamil Nadu, India.
[8*]Department of Biochemistry, Kongunadu Arts and Science College, Coimbatore - 641029, Tamil Nadu, India. Email: nirubamak@kongunaducollege.ac.in
[9]*Cancer Nanobiotechnology Laboratory, Department of Research, Saveetha College of Nursing (SCON), Saveetha Institute of Medical and Technical Sciences (SIMATS), Chennai, 602105, Tamil Nadu, India. Email: venkatbiochem11@gmail.com

Abstract:
Piper nigrum root, a traditional little-explored source of saponins, has promising therapeutic assets. Saponins are biologically active plant mixtures with antioxidant, antidiabetic, antimicrobial and anticancer assets. This study inspected saponin-rich extracts from Piper nigrum root, a traditional but scientifically unexplored source. The main components of saponin were identified using GC-MS analysis in addition phytochemical examination of the methanolic extracts. DPPH in addition FRAP assays were used to measure antioxidant and antidiabetic activities, accompanied by the inhibition of alpha-amylase also alpha-glucosidase. Antimicrobial and anticancer assays were done for the saponins. Using AutoDock Vina over Pyrx, it was found that the saponins are molecularly bonded to the target enzymes. The extract has been shown to have excellent activity in reducing FRAP and in scavenging DPPH (78.42 percent at 100 µg per ml). At 500 mg/mL, it inhibited both alpha-glucosidase and alpha-amylase by 79.31 and 75.14%, respectively. Furthermore, cytotoxicity assays clearly demonstrated a dose-dependent decrease in the viability of A431 skin cancer cells. Similar observations were obtained in mitochondrial membrane potential (MMP) assays using saponin. Docking studies have shown high affinity (up to - 9.1 kcal mol) for key enzymes, which is supported by multiple hydrogen-bond interactions. The dual therapeutic efficacy of P. nigrum root saponins highlights their viability as a natural alternative for the management of oxidative and diabetic diseases.

KEYWORD: Piper nigrum L, Saponins, GC-MS, Antioxidant, Anti-diabetic, Antimicrobial, Anticancer.

1. INTRODUCTION

Medicinal plants are packed with natural chemicals that can help with diseases concomitant to oxidative stress and metabolic problems, such as diabetes. For instance, Piper nigrum is known for its spice, but it also has impressive medicinal properties. It fights inflammation, regulates blood sugar, acts as an antioxidant, and even fights bacteria. Scientists have expended a lot of time reviewing the fruit, but no one has premeditated the roots thoroughly. Though, the root of P. nigrum is fuelled by saponins, a group of compounds that are imperative in glucose regulation and oxidative stress reduction. Saponins may backing in type 2 diabetes, a disease in which the blood sugar remains in elevation, and the body does not respond well to insulin. These compounds tackle diabetic enzymes, alpha-amylase besides alpha-glucosidase, which break down carbohydrates and cause blood sugar levels to rise [1]. Yet, drugs such as acarbose and miglitol lessen blood sugar levels but tend to have off-target effects on the digestive tract [2]. Hence, researchers are looking for plant alternatives to reduce these side effects. Oxidative stress plays a chief role in type 2 diabetes. It drives the body towards insulin resistance and upsets the efficiency of pancreatic beta cells [3]. Plant antioxidants can benefit to lessen oxidative stress. Piper nigrum roots are rich in phytochemicals, such as saponins, which deserve further investigation for their potential health benefits. Fourier Transform Infrared spectroscopy (FTIR) and Gas Chromatography Mass Spectrometry (GC-MS) techniques supports to categorize the existence of phytochemicals in the plant extract. GC-

MS is excellent for detecting volatile and semi-volatile compounds, such as phenols, flavonoids, terpenes, and alkaloids, which are significant for the pharmacological potency of a plant [4]. FTIR was used to discover the functional groups in charge for antioxidant activity. Together, these methods deliver a detailed picture of how these compounds act under physiological conditions [5]. Moreover, computational drugs have been discovered. Molecular docking allows the prediction of how plant compounds bind to target enzymes, alpha-amylase and alpha-glucosidase [6]. The major substance in the P. nigrum extract, piperine, has been reported for its toxicity through the induction of reactive oxygen species and hydroxyl radicals in rat testes [7,8]. Another study revealed that piperine promoted the stability of Aspergillus derived aflatoxin B1 in the liver [9]. To avoid these unsatisfactory effects, we improved this extract by eliminating piperine from the black pepper extract using a specific method [10]. Researchers are now using in silico studies to study the inhibition of these enzymes by natural molecules, providing a clue to their antidiabetic potential. This approach helps to rapidly identify new drug candidates and makes it easier to assess the therapeutic potential of numerous natural substances. Many researchers working on plant-based therapies for metabolic disorders have found that a combination of GC-MS profiling and molecular docking may reveal promising compounds for the management of type 2 diabetes and anticancer studies [11]. Although the medicinal properties of Piper nigrum are well known, the saponin fraction of the root has not been studied for its antioxidant, antidiabetic and anticancer properties. Hence, this study was directed to identify the phytochemicals of P. nigrum root extract using GC-MS and FTIR and evaluate their antioxidant and inhibitory potential of alpha-glucosidase enzyme. By mapping these bioactive components, this study opens new avenues for developing functional plant-based foods and medicines to help combat metabolic disorders.

2. MATERIALS AND METHODS

2.1Extract preparation of Piper nigrum root

The Piper nigrum roots were collected from Ooty. The roots were completely washed with sterile distilled water and dried under the shade. The roots were then homogenized to a fine coarse powder using an electric blender and the powder was extracted with methanol solvent in a soxhlet apparatus for 24 hrs. For the preparation of Methanolic extract, One gram (1g) of powdered material of both the leaves and roots were soaked in 20 mL of methanol and kept at shaking incubator at 50 60 rpm & 40ºC for 24 hrs. The mixture was then filtered with Whatmann No. 1 filter paper and the extract was collected [12]. The obtained extract from the solvent after evaporation was used for further study.

2.2 Qualitative Phytochemical Investigations

The extract was used for screening the following bioactive compounds: alkaloids, terpenoids, phenol and tannins, sugar, saponins, flavonoids, quinones, and proteins, according to the standard procedure described by [13].

2.3 Qualitative and Quantitative determination of Saponins

Preliminary phytochemical analysis was carried out for all the extracts as per standard methods described by [14]. Saponin extracts obtained by the above method was subjected to qualitative analysis for the confirmation of saponins.
Quantitative determination of saponin was done using the method by [15]. The saponin content was calculated as a percentage: % Saponin =Weight of Saponin / Weight of sample ×100

2.3.1 Gas Chromatography–Mass Spectrometry (GC-MS) Analysi

GC-MS analysis of the both methanolic extract of piper nigrum root were performed using a GC MS Clarus 500 Perkin Elmer system which comprised AOC- 20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument. GC was equipped with a fused capillary column Restek RtxR – 5, (30meter × 0.25 mm) (5% diphenyl / 95% dimethyl polysiloxane), running in electron impact mode at 70 eV. For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium (99. 999%) was used as carrier gas at a constant flow rate of 1ml/min.
Volume of 1.0 µl of each plant extract was injected to GC-MS which splits the components into 10:1 ratio and the injector temperature was maintained at 280°C, the ion-being 200°C. The oven temperature was set to 40°C (isothermal for 5 min), with an increase of 6°C/ min to 280°C, then ending with a 15min isothermal at 280°C. Mass spectra were taken at 70 eV; 0.5 seconds of scan interval and fragments from 40 to 550 Da. The total GC running time was 60 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas [16]. The bioactive chemicals were identified by comparing their mass spectra with those in the National Institute of Standards and Technology (NIST)database.

2.3.2 Analysis of Spectra

Using a UV-Visible Spectrophotometer (Shimadzu UV-1800), the saponin sample was scanned between 200 and 700 nm in wavelength, and the distinctive peaks were found and noted. FTIR was used to categorise functional groups linked to bioactive ingredients to assess the structural characteristics of saponins. A pellet was formed by encasing a 10-mL aliquot of the saponin extract in 100 mg of potassium bromide (KBr). An Agilent FTIR spectrophotometer was used to record FTIR spectra in the 400–4000 cm^{-1} range; each spectrum represented an average of 200 scans at a resolution of 4 cm^{-1} [17]. The attained spectra were analysed to govern the presence of characteristic functional groups such as hydroxyl, carbonyl, and ether linkages, which are commonly found in saponin structures.

2.4. Antioxidant activity

The antioxidant capacity of ethanolic extract of Piper nigrum root evaluated by five conventional in vitro assays: DPPH radical scavenging, hydroxyl radical scavenging, ferric reducing antioxidant power (FRAP), nitric acid along with ABTS antioxidant capacity.

2.4.1. DPPH Radical Scavenging Assay (RSA)

The antioxidant activity by unstable radical scavenging assay using stable DPPH and the absorbance was read at 517 nm against the control BHT. The antioxidant activity is based on the DPPH inhibition by saponin extract [18].

2.4.2. Hydroxyl radical scavenging assay

To test how well the saponin extract scavenges hydroxyl radicals, we set up the experiment like this: We mixed 850 µL of the saponin extract with 150 µL of a 4 mM hydrogen peroxide solution, all in a phosphate buffer (0.1 M, pH 7.4). After allowing the mixture to stand for 10 min, its absorbance was measured at 230 nm. Ascorbic acid was used as a positive control. Each reaction was performed in triplicate. The percentage inhibition was used Eq2.

$$\% \text{ Inhibition} = \frac{\text{Abs Control, 230-Abs Sample, 230}}{\text{Abs Control, 230}} \text{ x } 100 - \text{Eq2}$$

2.4.3. Ferric reducing/antioxidant power (FRAP) assay

The saponin extracts at different concentrations of 2.5 mL added with 2.5 mL of 1% potassium ferricyanide and 2.5 mL of 0.2 M sodium phosphate buffer made up the reaction mixture. Except sample all the reagents must be present in the control. After 20 min of incubation at 50 ºC, 2.5 mL of 10% (w/v) trichloroacetic acid was added, and the mixture was centrifuged for 10 min at 3000 rpm. Then, 1 mL of 0.1% FeCl3, 5 mL of deionised water and 5 mL of supernatant upper layer were mixed. The absorbance was measured at 700 nm against blanks with phosphate buffer and distilled water.

2.4.4. Nitric oxide (NO) scavenging activity

The interaction of sample with nitric oxide was assessed by the nitride detection method. Nitric oxide was generated from sodium nitroprusside and measured by Griess illusory reaction. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured at 540 nm against blank. 0.5 mL of the reaction mixture containing nitrite was removed, 1.0 mL of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min for completion of diazotisation and 1.0 mL of naphthyl ethylenediamine dihydrochloride was added, mixed well and allowed to stand for 30 min in diffused light. A pink colour chromophore was formed [19]. Rutin was used as a standard. The inhibition was calculated according to the equation:

$$\text{Percentage inhibition (\%)} = ((Ac - As)/Ac) \times 100$$

2.4.5. ABTS+ Radical Scavenging activity

The scavenging activity was determined by ABTS radical cation scavenging action by saponin extracts. ABTS+ solution was prepared into different concentration from 5-100 g/mL. The saponin mixture of sample extracts was added to the tubes and incubated for 6 min against the standard ascorbic acid. The absorbance was read at 734 nm [20].

2.5. Tests for Anti-diabetic activity

2.5.1. Assay for alpha-amylase inhibition

The ability of the saponin extract to suppress alpha-amylase activity was evaluated. A reaction mixture comprising 390 µL of 0.02 M phosphate buffer (pH 7.0), a positive control, or varying saponin extract quantities was pre-incubated with 10 µL of alpha-amylase at 37°C for 10 min. The reaction mixture was then incubated at 37°C for one hour after 10 µL of starch solution was added. After incubation, 0.1 mL of 1% iodine solution and 5 mL of distilled water were added, and the absorbance was measured at 565 nm.

2.5.2.Assay for alpha-glycosidase inhibition

The saponin extract's possible anti-diabetic efficacy was assessed using the alpha-glucosidase inhibition assay. 75 µL of alpha-glucosidase was pre-incubated at 37°C for 30 minutes with a reaction mixture that contained 225 µL of 80 mM phosphate buffer (pH 7.0), a positive control, or various saponin extract quantities. Placing the mixture stopped the reaction. After two minutes in a bath of hot water, let it cool. After adding 250 µL of glucose reagent, the mixture was allowed to sit at room temperature for ten minutes. At 510 nm, the absorbance was measured.

2.6. Tests for Anti-cancer activity

2.6.1. Cell culture, cytotoxic and MTT assays

The human skin cancer cell line (A431) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 370C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density

of 1x105 cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 370C, 5% CO2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethyl sulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 370C, 5% CO2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 370C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC50 was determined using GraphPad Prism software [21-22].

2.6.2. Cell culture and Measurement of mitochondrial membrane potential

A 431 (human skin carcinoma) cell line were cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin and 100 µg/ml streptomycin and maintained under an atmosphere of 5% CO2 at 37oC. The A 431 cells (1x105 cells/well) were plated to a 24 well plate in a DMEM growth medium for overnight at 37°C. The wells were washed with sterile PBS and treated with sample code – R at the concentration of 46.02 µg/ml in a serum free DMEM. The treated cells and control cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. The measurement of mitochondrial membrane potential for the treated and control cells was carried out according to the manufacturer's instruction. Briefly, the cells were incubated with 100 µl/well of JC-10 dye loading solution and plate was protected from light. The plate was incubated for 30–60 minutes in a 5% CO2 at 37 °C. After incubation, 100 µl of buffer B was added to each sample/well. Finally, the plate was centrifuged at 800 rpm for 2 minutes and the fluorescence was observed at 490/525 and 540/590 ratio. Green/Red fluorescence and for Hoechst dye excitation and emission at 350/461nm respectively. Images were analysed by ZEN Blue Software and recorded, and the expression of DCF intensity is measured using Image J software.

2.7. In-silico molecular docking studies

Molecular docking simulations were conducted using PyRx 0.8, an integrated virtual screening tool incorporating AutoDock Vina, to evaluate the interactions between the identified saponins and their respective target proteins. Target proteins were selected based on their reported biological relevance and retrieved from the Protein Data Bank (PDB). First, the protein structures were cleaned by removing any non-essential molecules, and AutoDock Tools and Open Babel were used for energy minimisation. The 3D structures of the saponins were obtained from PubChem and converted to PDBQT format using OpenBabel. For the docking simulations, I worked in PyRx using the binding sites reported in earlier studies. A grid box was set around each binding pocket to obtain the ligands in the right spot, and AutoDock Vina was run with an exhaustiveness of 8. After docking, each ligand-protein complex was ranked by binding affinity (in kcal/mol), with lower scores indicating stronger binding. To additional inspect, UCSF Chimera was used to scrutinise the molecular interactions, hydrogen bonds, hydrophobic contacts, and π-π stacking. These outcomes highpoint the interaction amongst saponins and their target proteins, supportive their impending roles in antioxidant and anti-diabetic therapies.

3. RESULTS

3.1. Preliminary Phytochemical Screening

In this study, we examined the phytochemical character of Piper nigrum root extract, concentrating on its bioactive assets. Our preliminary phytochemical tests on the methanolic extract revealed a wide range of secondary metabolites, listed in Table 1. Medicinal plants backing as vital reservoirs of bioactive mixtures, offering momentous potential in drug discovery and therapeutic presentations. Secondary metabolites such as alkaloids, flavonoids, tannins, and saponins play crucial roles in disease modulation, predominantly in oxidative stress related disorders and metabolic dysfunctions [23][24]. Notably, saponins, alkaloids, glycosides, tannins, and terpenoids were predominant suggesting potential pharmacological relevance. The great occurrence of these compounds aligns with previous studies on Piper species, underscoring their significance in medicinal chemistry [25].

Table 1. Preliminary Phytochemical screening of Piper nigrum root extract

Phytochemicals	Methanolic root extract of Piper nigrum
Alkaloids	+
Carbohydrate	+
Terpenoids	++

Steroids	++
Tannins	+
Saponins	++
Glycosides	+
Phenols	+

3.2. Qualitative and Quantitative determination of saponins

Saponins are amphiphilic glycosides with extensive assortment of pharmacological properties, including antioxidant, antimicrobial, and antidiabetic activities. In this study, we implemented standard tests including the foam test, Liebermann–Burchard test, sulfuric acid reaction to identify them saponin. All of them were positive (Table 2), designating the existence of saponins in the extract. When we measured the amount, the extract showed an average saponin concentration of 63±2.02%.

Table 2. The qualitative confirmation of saponins from Piper nigrum root extract

S.no	Test	Results (+' present)
1.	Extract + H2O (Foam)	+
2.	Extract + Conc.H2SO4	+
3.	Extract+Libermann-Burhard (Acetaldehyde, Conc.H2SO4, Chloroform)	+
4.	Extract + Formaldehyde, Conc.H2SO4	+
5.	Extract + Laphen (Conc.H2SO4, Cu2+)	+
6.	Extract + Salcovaski (Conc.H2SO4 & chloroform)	+

3.2.1 GC-MS analysis

GC-MS investigation of the butanol fraction from Piper nigrum root extract showing numerous phytochemical ingredients, exhibiting diverse pharmacological activities (Table 3), all categorized by retention time, molecular weight, and molecular formula of these mixtures indicate their structural and functional diversity, subsidising to the medicinal properties of Piper nigrum root. These findings align with the established role of plant secondary metabolites in improving adaptability to biotic and abiotic stresses.

Table 3. GC-MS Analysis of saponin from root of Piper nigrum

SNO	Phytochemical compound	Retention Time	Molecular Formula	Molecular weight	Pharmacological actions
1	Piperine	22.313	C17H19NO3	285	Analgesic
2	Cyclohexene	3.997	C10H16	136	Anti- Microbial
3	Piperidine	16.883	C14H17NO	215	Anti-bacterial, Analgesic Anti-inflammatory
4	Piperonyl-2,4-thiazolidinedione	20.98	C3H3NO2S	117	Anti-Diabetic
5	Piperonylpyrazole	18.43	C3H4N2	273	Anti-bacterial, Analgesic Anti-inflammatory
6	Propanedioic acid	3.150	C5H8O4	132	Antioxidant
7	1,6-octdien-3-ol-3,7-dimethyl	5.067	C10H18O	154	Anti-inflammatory
8	2-Methyl-1-ethylpyrollidine	5.696	C7H15N	113	Anti-bacterial
9	2-Isopropenyl-5-methylhex-4-enal	6.108	C10H16O	152	Anti-hyperglycemic
10	Pyrrolizin-1,7-dione-6-carboxylic acid	7.562	C9H11NO4	197	Anti-viral
11	7-epi-cis-sesquisabinene hydrate	8.35	C15H26O	222	Antioxidant
12	Phenol methoxy 4-propenyl	8.49	C10H12O2	164	Anti-inflammatory

13	Naphthalene,1, octahydro	8.95	C15H24	204	Anti-bacterial
14	Epiglobulol	9.18	C15H26O	207	Antioxidant, Anti-bacterial
15	Eicosanoic acid,2-ethyl ester	18.14	C27H50O6	470	Anti-inflammatory
16	Tetramethyl-6,7,8-tetra hydro-5H chromen	18.53	C13H20O2	208	Antioxidant
17	2H-1,2-benzoxazine 3-carbonitrile,2-cyclohexylocta	18.74	C17H28N2O	276	Anti-inflammatory
18	9-octadecenamide, n-butyl	20.751	C22H43NO	337	Anti- Microbial Antioxidant Anti-inflammatory

3.2.2 Spectral analysis

In this study, the UV-Vis spectrum of the Piper nigrum root (Figure 1) displayed strong absorbance between 200 and 400 nm. This is a classic sign of $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ electronic transitions, which usually appear in conjugated systems and compounds with carbonyl groups. This absorption pattern fits the expected profile of triterpenoidal saponins and related glycosides. These molecules are known for their strong UV absorption owing to their ring systems and sugar parts. The broad peak in the spectrum suggests that there is a mix of complex phytochemicals, all with overlapping electronic transitions.

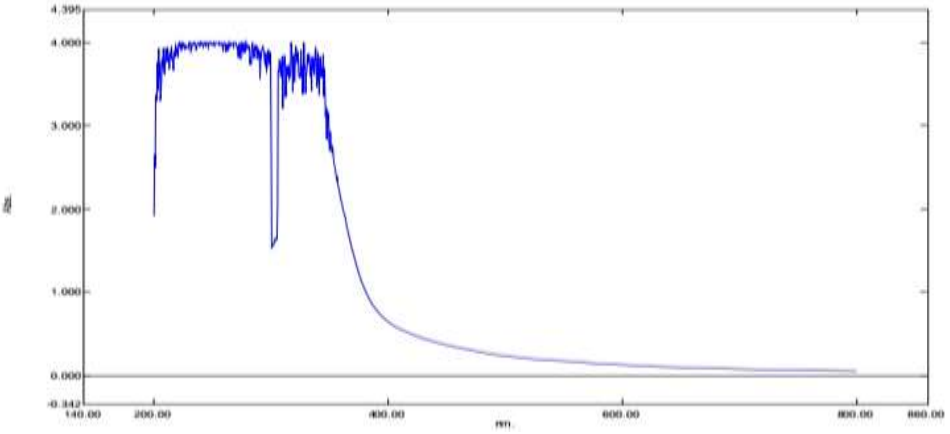
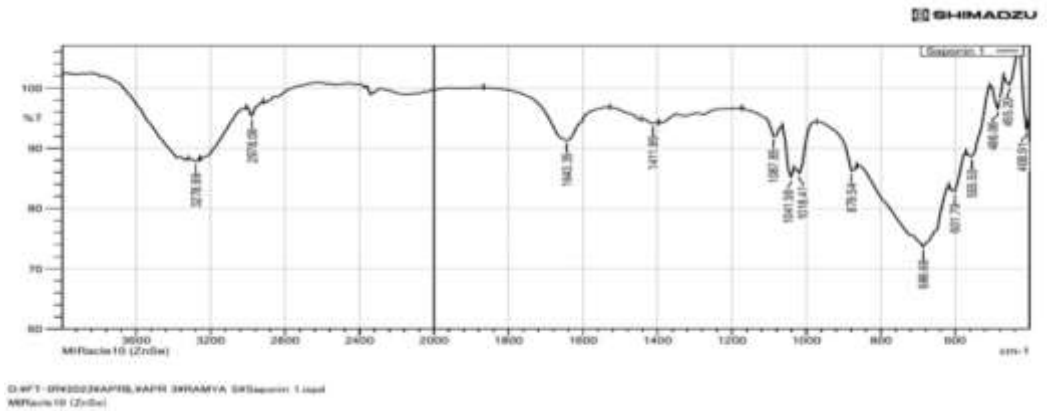


Figure 1. UV spectrum of saponins from root of Piper nigrum

(UV-Vis spectrum of the saponin-rich fraction from Piper nigrum root showed intense absorbance in the 11 200–400 nm range).

Along with UV-Vis analysis, FTIR spectroscopy helped identify the functional groups in the saponin fraction (Figure 2). In the FTIR spectrum, the O–H stretching vibrations from hydroxyl groups are observed as a broad absorption band at 3278 cm^{-1}, which is typical for saponin glycosides. The aliphatic $–CH_3$ and $–CH_2$ groups' C–H stretching vibrations are responsible for the peak at 2978 cm^{-1}.

a)



b)

S. No	Frequency (cm-1)	Functional groups/other details
1.	3278	COOH groups
2.	2978	Saturated CH
3.	1643	C=O
4.	1600-1630	C=C
5.	1400	C-H
6.	1000-1300	C-O
7.	700-800	CH2

Figure 2. FTIR of saponins from root of Piper nigrum

(a)The FTIR spectra of ethanolic extract of root of Piper nigrum displays typical absorption bands associated with various functional groups. b) A table listing the major peaks, corresponding bonds, and their related functional groups)

3.3 In vitro antioxidant activity

3.3.1 DPPH radical scavenging assay

In this study, we tested the ability of the saponin fraction from Piper nigrum roots to scavenge DPPH radicals and compared it that with of rutin, a well-known antioxidant. The results (Table 4) show how the saponin extract donates electrons or hydrogen atoms to neutralise DPPH radicals. This highlights the potential of the extract as a natural source of antioxidants.

Table 4: DPPH radical scavenging activity of saponin extract from root of Piper nigrum

Concentration (µl)	% Inhibition
10	54.92
50	59.84
150	60.66
250	68.03
350	72.95
500	73.77
750	78.69

3.3.2 Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay results, presented in Table 5, demonstrate the effectiveness of the extract in neutralising H_2O_2 and blocking the formation of more harmful radicals through the Fenton reaction. This indicates how the extract might help control oxidative stress by short-circuiting key steps in ROS production [26].

Table 5: Antioxidant assay of saponin extract from root of Piper nigrum

Concentration	Hydroxy radical activity	FRAP Assay	Nitric oxide scavenging activity	ABTS+ scavenging activity
10 µl	22.10%	30.00%	30.25%	25.00%
20 µl	35.00 %	43.10%	37.58%	33.20%
30 µl	54.25 %	53.25%	53.46%	47.50%
40 µl	72.45 %	68.31%	65.30%	58.70%
50 µl	81.40 %	77.60%	74.50%	70.39%

3.3.3 Ferric reducing/antioxidant power (FRAP) assay

The FRAP assay showed that as the concentration of the saponin extract from the Piper nigrum root increased, its ferric reducing power also increased. Basically, the more you add, the stronger the antioxidant effect. At 10 µL, the extract reached 30% activity, and at 50 µL, it increased to 77.6%. This is not far from ascorbic acid, which peaked at 87% (Table 5).

3.4 Anti-Diabetic Assay

3.4.1 Alpha-amylase enzyme inhibition assay

The α-amylase inhibition test showed something interesting: the saponin extract from Piper nigrum root really holds its own when it comes to antidiabetic activity. As the dose amplified, the inhibition also amplified, opening at 30% with 10 µL and reaching 77.6% at 50 µL. This is not far behindhand ascorbic acid, which stretched 87% at the same level (Table 6). As α-amylase plays a major role in breaking down carbohydrates, its inhibition can help manage postprandial blood sugar spikes by slowing down starch digestion. The extract kept up with the standard almost all the way, which points to its real promise as a natural α-amylase inhibitor.

Table 6: Anti-diabetic assay of saponin extract from root of Piper nigrum

Concentration	Alpha-amylase enzyme inhibition assay	Alpha- glycosidase enzyme inhibition assay
10 µl	30.00%	28.00%
20 µl	43.10%	33.50%
30 µl	53.25%	44.38%
40 µl	68.31%	61.18%
50 µl	77.60%	69.42%

3.4.2 Alpha-glycosidase enzyme inhibition assay

The α-glucosidase inhibition assay showed that the Piper nigrum root saponin extract blocked the enzyme in a dose-dependent manner. Even at the lowest dose tested (10 µL), the extract inhibited the enzyme by 28%. At the highest dose (50 µL), the inhibition increased to 69.42%. Ascorbic acid performed slightly better, with 81% inhibition at the same dose; however, the extract still performed well across all concentrations (Table 6).

3.5. In vitro cytotoxic studies

3.5.1. MTT assay

The cytotoxic effect of saponins from piper nigrum root was assessed against A431 cells. After 24 h incubation with different concentration of saponins potentially decreased the cell viability of A431 cells with the IC50 value of 46.02 g/mL. The results (Figs. 3 and 4) clearly indicated that the varying concentration (6.5, 12.5, 25, 50 and 100 g/mL) of saponin treatment was significantly inhibit the proliferation of A431 cells in a dose dependent manner. The cellular uptake and cytotoxic effect of nanoparticles depend largely on their physico chemical properties such as size, surface charge and aggregation. Synthesized saponin efficient cellular entry through diffusion or membrane channels. This extract enhanced interaction with A431 cells, resulting in the dose-dependent reduction in cell viability observed in the cytotoxicity assay, thereby confirming the biological activity of the saponins.

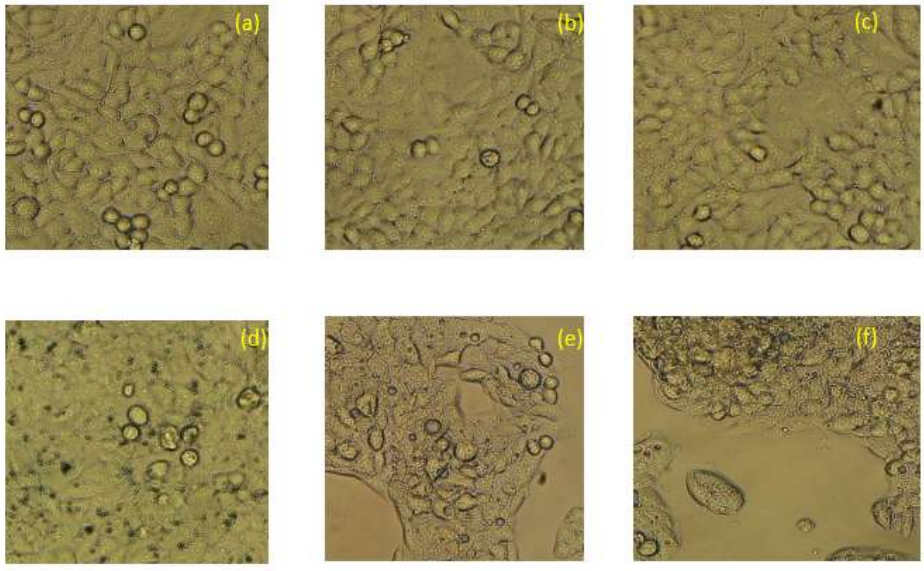


Figure 3. Microscopy images showing cytotoxic effect of saponins from P.Nigrum root on A431cells at different concentrations: (a) control, (b) 6.5, (c) 12.5, (d) 25, (e) 50 and (f) 100 g/mL

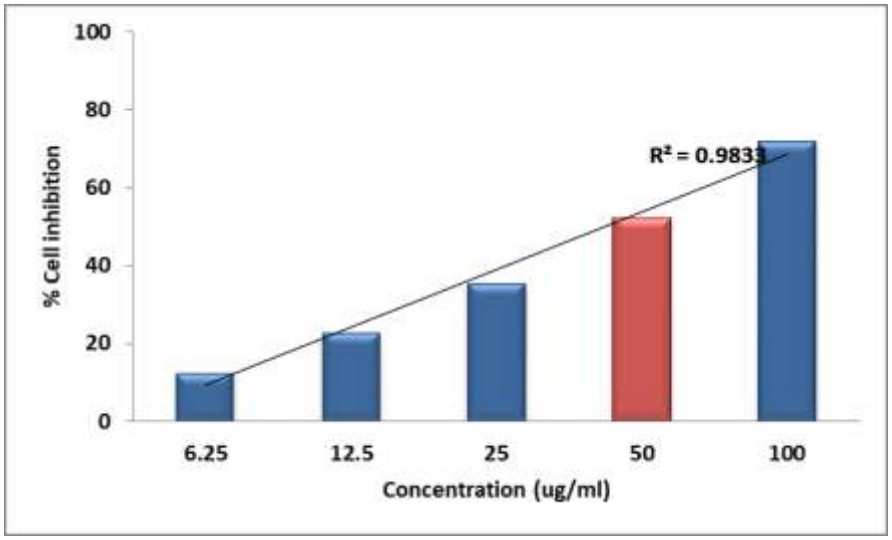


Figure.4. Cytotoxic effect of different concentration of saponins

3.5.2. MMP asssy

In the present study, A431 cells treated with saponin showed both green and red fluorescence, indicating loss of mitochondrial membrane potential, whereas control cells mainly exhibited normal fluorescence (Fig. 5).

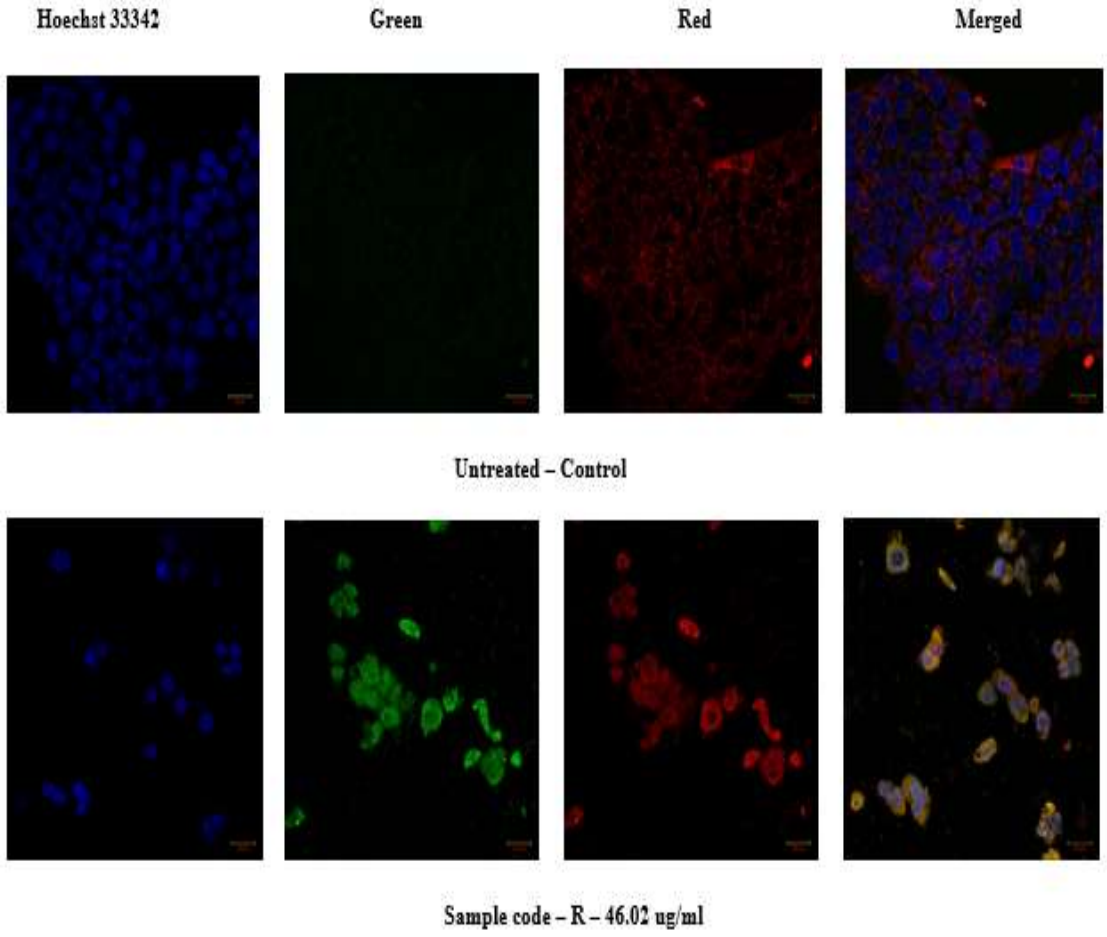


Figure 5. Effects of saponin from piper nigrum root treatment after 24 h on mitochondrial membrane potential of A431 cells assessed by JC-10 dye staining; (a) control and (b) 46.02 g/mL

3.6 In - silico Molecular Docking Studies

Overall, the variety of these compounds suggests that the extract acts on multiple molecular targets, making it a good candidate for molecular docking studies to check binding affinity and support drug discovery. For this study, six key pharmacological targets were chosen because they are related to the main bioactivities of the extract: analgesic,

antimicrobial, anti-inflammatory, antidiabetic, antiviral, and antioxidant effects. Identifying the active sites in these target proteins is crucial for structure-based drug design and determining how the compounds in the extract might interact with them. The μ-opioid receptor is central to pain control. Drug developers focus on this receptor because it is key to managing pain. Its active site, comprising Asp147, Tyr148, His297, Met151, Trp293, Ile322, and Phe338, regulates ligand binding and initiates receptor activity (Table 7) [27]. Researchers can work with its crystal structure (PDB ID 4DKL), which makes docking and interaction studies easier. ii. Penicillin-Binding Proteins (Antimicrobial Target): PBPs keep bacteria alive by building their cell walls. Their active sites, Ser337, Lys545, and Ser389, drive their transpeptidase function (Table 7). These spots are where β-lactam antibiotics act. Scientists have pinned down the structure with the PDB ID 1TVF. iii. Cyclooxygenase-2 (COX-2) (Anti-inflammatory Target): COX-2 converts arachidonic acid into prostaglandins, which induces the active site, Arg120, Tyr355, and Ser530, is located inside a hydrophobic channel, where NSAIDs exert their effects (Table 7). The structure is available under the PDB ID 1CX2. iv. Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) (Antidiabetic Target): PPAR-γ is a nuclear receptor that controls glucose and fat metabolism. Its Y-shaped binding pocket, with Ser289, His323, and Tyr473, is crucial for capturing ligands and activating the receptor (Table 7). Its structure can be found in PDB ID 2PRG, which is a significant aid for anyone designing antidiabetic drugs. RNA-Dependent RNA Polymerase (RdRP) (Antiviral Target): RdRP drives viral genome replication, especially in RNA viruses such as SARS-CoV-2. It relies on Asp618, Asp760, and Asp761, all tucked inside motifs A–F, to get its job done (Table 7). The PDB structure 7BV2 proposals researchers with a perfect target for developing antiviral inhibitors that block RNA synthesis. vi. Glutathione Peroxidase (Antioxidant Target): This enzyme shields cells by neutralizing hydrogen peroxide, lipid peroxides thereby keeping redox balance in check. Its active site features selenocysteine (Sec46), Gln81, and Trp136, which work together in redox cycling [28] (Table 7). The enzyme structure documented under PDB ID 213Y.

Table 7: Identification of Target receptors and active sites

Target	Active Site	Commonly used PDB ID
μ-Opioid Receptor (Analgesic Target)	Asp147, Tyr148, His297, Met151, Trp293, Ile322, and Phe338.	4DKL
Penicillin-Binding Proteins (Antimicrobial Target)	Ser337, Lys545, and Ser389.	1TVF
Cyclooxygenase-2 (COX-2) (Anti-inflammatory Target)	Arg120, Tyr355, and Ser530.	1CX2
PPAR-γ (Anti-diabetic Target)	Ser289, His323, and Tyr473.	2PRG
RNA-Dependent RNA Polymerase (Antiviral Target)	Asp618, Asp760, and Asp761	7BV2
Glutathione Peroxidase (Antioxidant Target)	Selenocysteine (Sec46), Gln81, and Trp136.	213Y

Target Active Site Commonly used PDB ID μ-Opioid Receptor (Analgesic Target) Asp147, Tyr148, His297, Met151, Trp293, Ile322, and Phe338. 4DKL Penicillin-Binding Proteins (antimicrobial targets) Ser337, Lys545, and Ser389. 1TVF Cyclooxygenase-2 (COX-2) (Anti-inflammatory Target) Arg120, Tyr355, and Ser530. 1CX2 PPAR-γ (antidiabetic target) Ser289, His323, and Tyr473. 2PRG RNA-Dependent RNA Polymerase (Antiviral Target) Asp618, Asp760, and Asp761 7BV2 Glutathione Peroxidase (Antioxidant Target) Selenocysteine (Sec46), Gln81, and Trp136. 213Y.

Table 8: Target Receptor and Molecule Allocation

Target	Molecule allocation			
μ-Opioid Receptor (Analgesic Target)	Piperine	Piperidine	Piperonylpyrazole	-
Penicillin-Binding Proteins (Antimicrobial Target)	Cyclohexene	Piperidine	Piperonylpyrazole	Epiglobulol
Cyclooxygenase-2 (COX-2) (Anti-inflammatory Target)	Piperidine	Piperonylpyrazole	1,6-octdien-3-ol-3,7-dimethyl	-
PPAR-γ (Anti-diabetic Target)	Piperonyl-2,4-thiazolidinedione	-	-	-
RNA-Dependent RNA Polymerase (Antiviral Target)	Pyrrolizin-1,7-dione-6-carboxylic acid	-	-	-

Glutathione Peroxidase (Antioxidant Target)	Propanedioic acid	Epiglobulol	-	-

3.5.1 Binding Affinity studies

i. Anti-analgesic docking profiles:

We executed molecular docking studies to describe how Piperine, Piperidine, and Piperonylpyrazole interact with the μ-opioid receptor. For this, we used AutoDock Vina via PyRx. Before docking, we optimised the receptor and screened all three ligands to compare their binding affinities. The results showed a clear difference in binding strengths, with piperonylpyrazole standing out as the strongest binder. The binding energies were as follows: piperine, −3.4 kcal/mol; piperidine, −6.5 kcal/mol; and piperonylpyrazole, −7.7 kcal/mol.

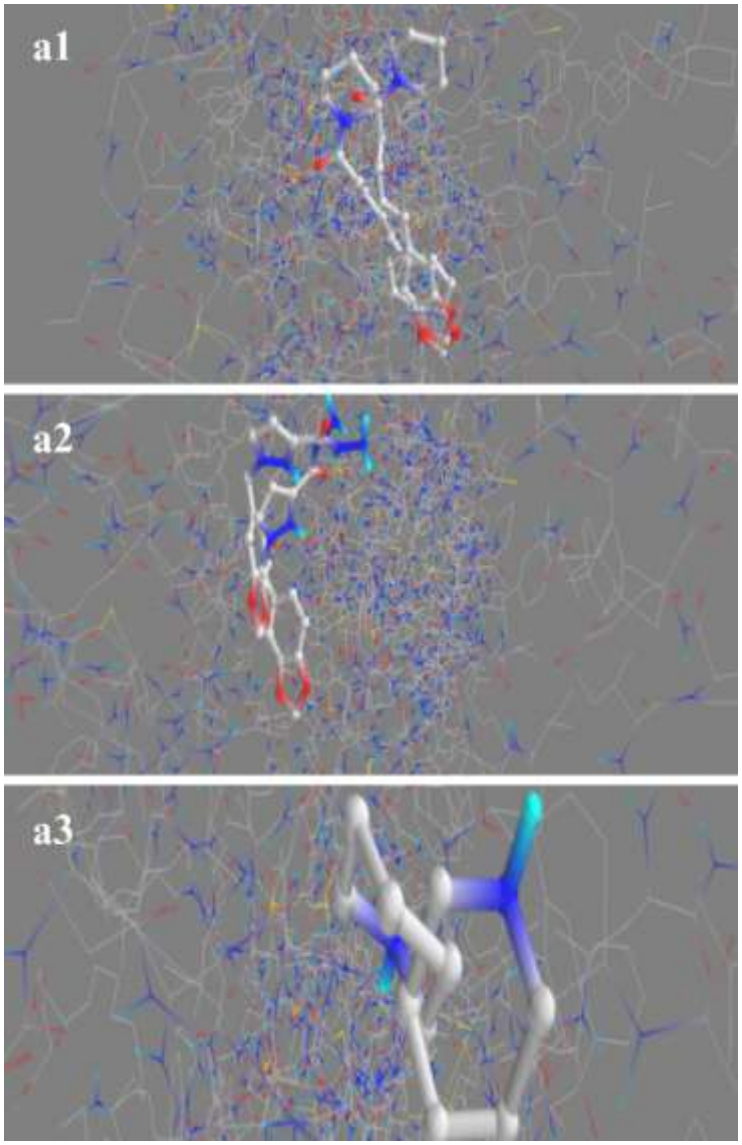


Figure 6. a1) Target protein against Piperine: a2) Target protein against Piperidine; a3) Target protein against Piperonylpyrazole

ii. Anti-inflammatory docking profiles

We performed molecular docking study to determine the interactions of certain ligands with Penicillin-Binding Proteins (PBPs), which are key targets for antibiotics. First, we optimised the PBP structure using AutoDock Vina in PyRx to ensure that the molecules would fit together properly in the docking simulations. We screened several ligands using a grid centred at X: 7.0908, Y: 8.7131, Z: 24.6790 to cover the main area where binding should occur. Among all the ligands tested, only cyclohexene showed promise. It demonstrated a binding energy of −6.4 kcal/mol, indicating a moderate but solid affinity for the protein.

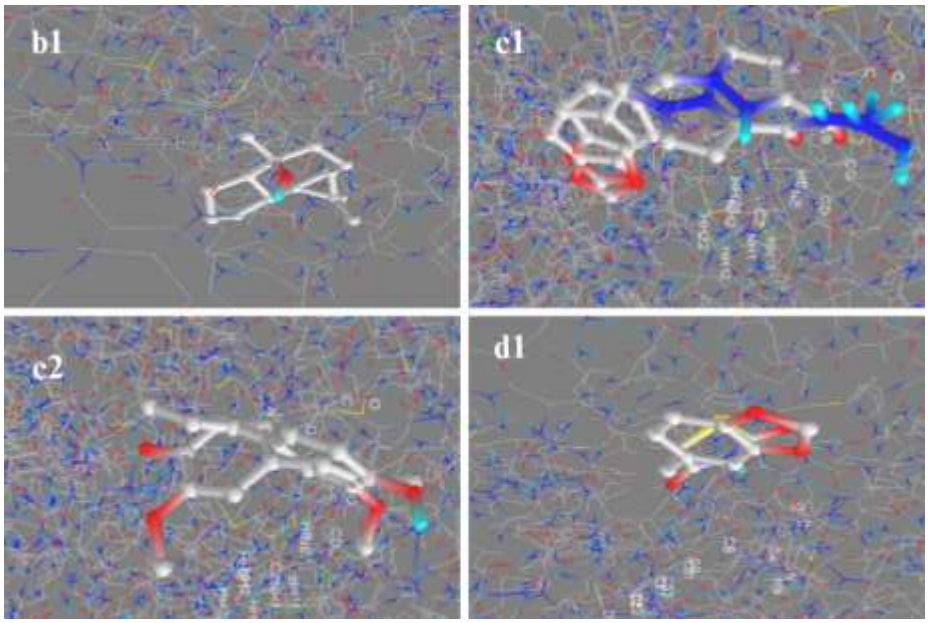


Figure 7. b1) Target protein against Cyclohexane; c1) Target protein against Piperidine; c2) Target protein against Piperonylpyrazole; d1) Target protein against Piperonyl-2,4-thiazolidinedione

4. DISCUSSION

Medicinal plants pack a punch when it comes to bioactive compounds, and they've shaped the way we discover and use new drugs. Taking their secondary metabolites—alkaloids, flavonoids, tannins, saponins. They help fight off illnesses, especially ones secured to oxidative stress or metabolic issues. If we look at saponins, studies keep finding saponins in abundance in Piper nigrum (that's black pepper). So, no surprise that Piper nigrum roots turn out to be overloaded with bioactive stuff, especially saponins. Other research backs this up and shows that plants rich in saponins often have strong antioxidant and metabolic benefits. Most saponins in dicot plants like Piper nigrum are triterpenoidal [29-35].

The piperine shows up at a retention time of 22.313 and is well-known for its pain-relieving power. Studies say piperine works by messing with inflammation and pain signals travel in your body. And found right alongside all those saponins, it proves how different plant chemicals can team up and make the whole mix even stronger. There are other interesting compounds—piperidine (16.883) and cyclohexene (3.997). People have used black pepper for ages to fight infections, and cyclohexene aids by damaging bacterial cell membranes and calming inflammation [36]. Piperidine is multitasker; it eases pain, reduces inflammation, and does even more. From a medical angle and remarkable. And worth mentioning: 2-isopropenyl-5-methylhex-4-enal (6.108) and piperonyl-2,4-thiazolidinedione (20.980). Both sparkle when it comes to managing blood sugar. Thiazolidinediones, stimulate the body's peroxisome proliferator-activated receptors (PPARs), which are key for handling glucose. So, it all comes together—black pepper isn't just a spice; it's packed with complexes that work together to support your health. The piperonyl-2,4 thiazolidinedione and propanedioic acid (Retention Time: 3.150), 7-epi-cis sesquisabinene hydrate (8.350), and epiglobulol (9.180). These compounds give its medicinal punch. Antioxidants tackle reactive oxygen species (ROS), help protect against oxidative stress and all the problems that come with it [37-40]. Epiglobulol, a sesquiterpene alcohol stands out studies show it's a powerful antioxidant that shields cells from oxidative damage. The anti-inflammatory effects get a lift from phenol methoxy 4-propenyl (8.490) and eicosanoic acid, 2-ethyl ester (18.140). Phenolic compounds are famous for calming inflammation by blocking enzymes like cyclooxygenase (COX). Eicosanoic acid derivatives, such as the 2-ethyl ester, can alter the way cell membranes are built and function. This tweak in lipid makeup can ramp down the production of inflammatory chemicals like prostaglandins and leukotrienes.

On the antiviral front, pyrrolizin-1,7-dione-6-carboxylic acid (7.562) displays promise. Pyrrolizine derivatives have reactive structures that block viruses from replicating- by attaching to the genetic material or by shutting down viral polymerases. Their oxygen and nitrogen atoms make stick to viral targets more simply. 9-octadecenamide, n-butyl (20.751), a fatty acid amide with benefits: anti-inflammatory, antioxidant, and antibacterial effects. These amphiphilic molecules have a knack for interacting with both nuclear receptors and membrane enzymes. Their unique features like chain length and unsaturation make cell membranes more flexible and improve signalling. Because of this, fatty acid amides look like solid candidates for new multitarget therapies.

The UV-Vis and FTIR tests are quick, gentle on samples. They help pinpoint different chromophores and functional groups linked to bioactive mixtures. UV-Vis, checks compounds soak up light at various wavelengths, scanning various types of phytochemicals exist. When there is no much absorption between 2500 and 1900 cm^{-1}, it means there aren't cumulated π-bond systems (alkynes or allenes). But a clear signal at 1643 cm^{-1} pops up—this is from C=O stretching, probably from ester and carboxylic groups in saponin aglycones or similar compounds. The bending vibrations at 1411

and 1375 cm^{-1} these displays as symmetric, asymmetric $–CH_2$ deformations. There's a peak at 1261 cm^{-1}, C–O–H bending inside the sugar part of the molecule. At 1076 cm^{-1}, a sharp band marks C–O–C stretching, which shows ether linkages in glycosidic bonds. All the spectral evidence points to the saponins we pulled from the extract being triterpenoid glycosides [34,41].

The antioxidants, the focus usually lands on how well a compound blocks free radicals. The DPPH assay is a simple way to check. DPPH is a stable free radical, deep violet in color, with a big absorbance at 517 nm because of its unpaired electron. When an antioxidant like a polyphenol or a saponin meets DPPH, it donates hydrogen atom. The DPPH then turns into a reduced, non-magnetic molecule, and you can see the colour shift from purple to yellow. Bigger colour changes mean stronger antioxidant activity [42].

Our bodies are always manufacture free radicals and other reactive oxygen species (ROS) during normal metabolism. Usually, these molecules aid with cell signaling and balance. But if ROS levels get too high. That's when oxidative stress kicks in, damaging lipids, proteins, and DNA. Lipid peroxidation is one of the key problems ROS attack polyunsaturated fatty acids, producing malondialdehyde (MDA) as a byproduct. MDA is basically the gold standard for tracking oxidative stress and lipid damage. The TBARS assay measures MDA to tell how much oxidative damage cells are dealing with. In this study, we looked at how well a saponin-rich extract from Piper nigrum root could scavenge hydrogen peroxide (H_2O_2), a particularly troublesome ROS, and we compared its performance to ascorbic acid, a well-known antioxidant.

The results show as the dose went up, the saponin extract's reducing power kept increasing, probably because of those glycosidic and polyphenolic groups. At mid-level doses, the extract worked almost as well as ascorbic acid at lowering ferric ions, which points to its strong ability to fight oxidative stress. This trend matched what saw with the DPPH and hydrogen peroxide scavenging assays. When looked at nitric oxide scavenging, the saponin extract again displayed a solid, dose-dependent effect. At 10 µL, it inhibited nitric oxide by 30.25%, right in line with the usual standard of 30%. At 50 µL, inhibition shot up to 74.5%. Ascorbic acid topped out at 81% (see Table 5 for details). Basically, saponins are good at mopping up nitric oxide radicals. They seem to do this by handing over electrons or hydrogen, which helps neutralize these reactive nitrogen species. When concentration of the extract, its ability to scavenge nitric oxide goes up too. That lines up with how it performs in other antioxidant tests, like the $ABTS^+$ radical scavenging test, where the same trend pops up.

The nitric oxide scavenging test, the saponin extract blocked about 30.25% of radicals at just 10 µL, which matches the usual benchmark of 30%. Bump it up to 50 µL, and you get 74.5% inhibition. For comparison, ascorbic acid hit 81% (see Table 5). This shows saponins work well as natural NO scavengers, especially at higher doses, and their antioxidant punch isn't limited to just one type of radical [36].

The saponins from Piper nigrum root, they might help keep blood sugar stable after meals by slowing down how fast carbs break down since α-glucosidase handles carb digestion and glucose absorption [43-46]. That supports the idea that saponins may help manage diabetes. GC-MS profiling of the extract revealed a mix of phytochemicals, with retention times between 3.15 and 22.313 minutes. That means got both low- and high-polarity molecules in there. One standout is piperine (RT: 22.313 min), an alkaloid famous for boosting the effects of other drugs by blocking cytochrome P450 enzymes. Plus, it's got pain-relieving properties, making it useful for both treatment and drug delivery.

There's cyclohexene (RT: 3.997 min), which is known to fight bacteria probably by messing with their membranes. The extract also packs piperidine and its derivative, piperonyl pyrazole (RT: 16.883 and 18.430 min), which have antibacterial and anti-inflammatory effects, like reducing swelling and easing discomfort.

On the antioxidant front, compounds like propanedioic acid (RT: 3.15 min) and 7-epi-cis sesquisabinene hydrate (RT: 8.35 min). These help tackle oxidative stress, which is a big deal in chronic diseases like diabetes and neurodegeneration. Another interesting find is 2-isopropenyl-5-methylhex-4-enal (RT: 6.108 min), which fits with about aldehyde derivatives helping to control blood sugar. And pyrrolizin-1,7-dione-6-carboxylic acid (RT: 7.562 min) and epiglobulol (RT: 9.18 min) add antiviral and antibacterial powers to the mix. All in all, this saponin extract brings a lot to the table—antioxidant, antidiabetic, antibacterial, and even antiviral activities [47].

Saponins are secondary metabolites that belong to a diverse group of compounds with high chemoprevention potential which have multidirectional effects on various processes related to the promotion and progression of cancer in which they can inhibit proliferation and induce the apoptosis of tumor cells, reduce their invasive activity, also it can influence the cancer cells through the modulation of intracellular signalling pathways associated with the oxidative stress and inflammation [48-51].

In this study, the antitumor activity of saponin as in vitro was evaluated by using MTT cytotoxicity assay. The results of the present study demonstrated that standard saponin are cytotoxic to human cancer cells in which they decreased the viability in a dose-dependent manner after 48 hr treatment with IC50 value 46.02 µg/ml respectively. In addition, the data showed that cisplatin has potent cytotoxic activity against human cancer cells with an IC50 value of 3.8 µg/ml. Other study showed that crude saponin isolated from Platycodi Radix induces apoptosis in HT-29 colon cancer cell line with

IC50 value 37.07 µg/ml in 24 h treatment [52]. Also in this regard, saponins isolated from Solanum trilobatum leaf is prefer to retally cytotoxic to human larynx carcinoma cells in a dose-dependent manner with an IC50 value of 1 mg/ml after 24 hr treatment [53]. Moreover, Lu et al. study showed that the viability of human breast cancer cells (MCF-7) was decreased when Rhizoma Paridis saponins concentration was raised in a dose-dependent manner with an IC50 value of 71.2 µg/ml after 48 hr treatment [54-55].

Docking tests were conducted using the 3D models of these compounds that were acquired from ChemSketch. As binding strength went up, the numbers dropped piperonyl pyrazole ended up on top. The imitations flagged a handful of key residues in the receptor's binding pocket: Asp147, Tyr148, His297, Met151, Trp293, Ile322, and Phe338. These amino acids do most of the heavy lifting when it comes to stabilizing the ligands. For the docking, we set the grid centre at X: −25.6712, Y: −8.4961, Z: −9.6283, targeting for the best fit. Piperine landed someplace in the middle, showing moderate affinity [44,56]. It trusted mostly on hydrogen bonds and hydrophobic contacts, but its binding energy was weak, so it just didn't stay put in the receptor. Piperidine did better, forming strong hydrophobic contacts with Phe338 and Ile322, which really anchored it in place. Piperonylpyrazole, though, was clearly the star. It formed especially stable connections with Trp293 and His297 two residues that are serious for stabilizing µ-opioid ligands. The compound's conjugated structure and heterocyclic core seemed almost tailor-made for the active site. Its aromatic groups and hydrogen bond donor/acceptor features kicked its stabilizing power up another notch, making it a promising candidate for µ-opioid receptor modulators. Docking data and molecular images (see Figure 3) showed that piperonyl pyrazole fit the receptor pocket tightly and interacted aggressively. If we tweak its structure further, we might end up with even more potent and targeted opioid receptor treatments.

This study drives how important hydrophobic and hydrogen bonding interactions are in designing µ-opioid receptor ligands. Piperonylpyrazole stands out as strong option for more drug development and lab testing. Looking closer at the structures, the cyclohexene got cozy with key amino acids in the binding pocket Lys545 and Ser389 using both hydrophobic contacts and likely hydrogen bonds. These interactions helped stabilize cyclohexene in the receptor. The docking images (Figure 4) clearly display cyclohexene settling into the hydrophobic cavity of the PBP, thanks to its aliphatic structure. The other ligands didn't bind as well, which tells their structures could use some redesigning to lift their protein interactions [45].

We refined the protein and docked for the anti-inflammatory target, cyclooxygenase-2 (COX-2), using the same AutoDock Vina setup in PyRx. The grid centre here was at X: 28.1234, Y: 21.2462, Z: 19.4329. Piperonylpyrazole topped the chart again with a score of −7.1 kcal/mol, while piperidine lagged behind at −5.2 kcal/mol. Digging into the binding pocket, we saw that piperonyl pyrazole formed strong bonds with Arg120, Tyr355, and Ser530 amino acids that play big roles in stabilizing ligands and are crucial for NSAID action. It made hydrophobic contacts and hydrogen bonds, particularly with Ser530 and Tyr355, locking in a stable complex. It formed electrostatic contacts with Arg120, adding even more stability (see Figure 4) [56,57]. Piperidine, by comparison, just didn't interact as strongly no surprise its binding score was lower. Switching gears to the anti-diabetic target, peroxisome proliferator-activated receptor gamma (PPAR-γ), we optimized docking at X: 59.3325, Y: -30.1810, Z: 19.9924 [48]. Here, piperonyl-2,4-thiazolidinedione stood out with a high binding affinity of −5.4 kcal/mol. Docking data showed it connected steadily with three key residues: Tyr473, His323, and Ser289. It relied on hydrophobic interactions with Tyr473 and hydrogen bonds with Ser289 and His323, which helped keep the ligand stable in the receptor pocket (Figure 4). All things considered, piperonyl-2,4-thiazolidinedione looks like solid Option for PPAR-γ modulation and could help with insulin sensitivity and glucose regulation.

CONCLUSION

We saw both FTIR and GC-MS on saponin extract from Piper nigrum root to figure out which bioactive chemicals are there. No one's really observed at the chemical profile of the edible root before, so this was new ground. We found a mix of phytochemicals, each with their own possible therapeutic uses. The extract turned out to have strong antioxidant and antidiabetic effects, which means it could help treat metabolic diseases and fight oxidative stress. Furthermore, the saponin exhibited significant cytotoxic activity against A431 cells through a mitochondrial-mediated apoptotic pathway. These findings indicate that saponin from P. Nigrum root extract possess promising anticancer potential against A431 cancer cells. Above that, our molecular docking tests showed that some of these compounds stick well to key therapeutic targets so there's real potential here for drug development. Even so, we need more biological tests and clinical trials before we know for sure if these discoveries will lead to safe, effective medicines.

Acknowledgment

We thank our institution and laboratory team for their support and assistance during this research work.

Conflict of interest

The authors have no conflicts of interest to declare.

Funding

This study did not receive any financial support.

REFERENCES

1. J. Upadhyay et al., "Pharmacotherapy of type 2 diabetes: An update," Metabolism., vol. 78, doi: 10.1016/j.metabol.2017.08.010, pp. 13–42 (2018).
2. M. S. H. Kabir et al., "Phytochemical screening, Antioxidant, Thrombolytic, α-amylase inhibition and cytotoxic activities of ethanol extract of Steudnera colocasiifolia K. Koch leaves," J. Young Pharm., vol. 8, no. 4, doi: 10.5530/jyp.2016.4.15, pp. 391–397 (2016).
3. H. P. V. Rupasinghe, S. Sekhon-Loodu, T. Mantso, and M. I. Panayiotidis, "Phytochemicals in regulating fatty acid β-oxidation: Potential underlying mechanisms and their involvement in obesity and weight loss," Pharmacol. Ther., vol. 165, doi: 10.1016/j.pharmthera.2016.06.005, pp. 153–163 (2016).
4. J. Liang, J. Sun, P. Chen, J. Frazier, V. Benefield, and M. Zhang, "Chemical analysis and classification of black pepper (Piper nigrum L.) based on their country of origin using mass spectrometric methods and chemometrics," Food Res. Int., vol. 140, no. May, doi: 10.1016/j.foodres.2020.109877, p. 109877 (2021).
5. G. J. Mohammed, A. M. Omran, and H. M. Hussein, "Antibacterial and phytochemical analysis of piper nigrum using gas chromatography – mass spectrum and fourier-transform infrared spectroscopy," Int. J. Pharmacogn. Phytochem. Res., vol. 8, no. 6, pp. 977–996 (2016).
6. F. Tasleem, I. Azhar, S. N. Ali, S. Perveen, and Z. A. Mahmood, "Analgesic and anti-inflammatory activities of Piper nigrum L.," Asian Pac. J. Trop. Med., vol. 7, no. S1, doi: 10.1016/S1995-7645(14)60275-3, pp. S461–S468 (2014).
7. Jancyhelan, J., Thirumurugan, A., Alagumanian, S., & Vignesh, K. (2026). Eco-friendly biosynthesis of silver nanoparticles using marine algal extract and their biomedical applications. *Results in Chemistry*, 103606.
8. Chinta G, Coumar MS, Periyasamy L. 2017. Reversible testicular toxicity of piperine on male albino rats. Pharmacogn Mag. 13(Suppl. 3):S525–S532.
9. S. Muthu, A.B. Altemimi, M. Lakshmikanthan, K. Krishnan, Q.H. ALKaisy, F. H. Awlqadr, M.A. Hesarinejad, Phycocolloids from Sargassum microcystum: immunomodulatory and antioxidant activities of alginic acid and fucoidan, Food Hydrocoll. Health 7 (2025) 100209, https://doi.org/10.1016/j.fhfh.2025.100209.
10. Allameh A, Saxena M, Biswas G, Raj HG, Singh J, Srivastava N. 1992. Piperine, a plant alkaloid of the Piper species, enhances the bioavailability of aflatoxin B1 in rat tissues. Cancer Lett. 61(3):195–199.
11. Sriwiriyajan S, Ninpesh T, Sukpondma Y, Nasomyon T, Graidist P. 2014. Cytotoxicity screening of plants of genus Piper in breast cancer cell lines. Trop J Pharm Res. 13(6):921.
12. Ayyadurai, T., Mohan, P. K., Annamalai, A., Kamaraj, C., & Waheeb, M. Q. (2026). Biogenic silver nanoparticles synthesis from Guilandina bonduc L. seed kernel extract with antibacterial, antioxidant and anticancer potential against ovarian Cancer cells. Results in Chemistry, 103246.
13. M. Lakshmikanthan, S. Muthu, K. Krishnan, A.B. Altemimi, N.N. Haider, L. Govindan, J. Selvakumari, Z.T. Alkanan, F. Cacciola, Y.M. Francis, A comprehensive review on anthocyanin-rich foods: insights into extraction, medicinal potential, and sustainable applications, Journal of Agriculture and Food Research. 17 (2024) 101245, https://doi.org/10.1016/j.jafr.2024.101245.
14. GA Ayoola, HAB Coker, SA Adesegun, AA Adepoju-Bello, K Obaweya1, EC Ezennia1, TO Atangbayila. Phytochemical Screening and Antioxidant Activities of Some Selected Medicinal Plants Used for Malaria Therapy in Southwestern Nigeria. Tropical Journal of Pharmaceutical Research, September 2008; 7 (3): 1019-1024.
15. SM Astuti, MAM Sakinah, RBM Andayani, Awalludin Risch. Determination of Saponin Compound from Anredera cordifolia (Ten) Steenis Plant (Binahong) to Potential Treatment for Several Diseases. Journal of Agricultural Science Vol. 3, No. 4; December 2011.
16. Ejikeme C, CS Ezeonu, Augustine N. Eboatu. Determination of physical and phytochemical constituents of some tropical timbers indigenous to niger delta area of nigeria. European Scientific Journal June 2014 edition vol.10, No.18 ISSN: 1857 – 7881 (Print) e - ISSN 1857- 7431.
17. S. Balasubramanian, D. Ganesh, Poonam Panchal, Mohammad Teimouri and Surya Narayana V. V. S. GC-MS analysis of phytocomponents in the methanolic extract of Emblica officinalis Gaertn (Indian Gooseberry). Journal of Chemical and Pharmaceutical Research, 2014, 6(6):843-845
18. J Emmanuel, F Ngasoh, A Bello, V Anye, A Onwualu. Phytochemical Study of Some Plant Extracts to Assess their Synergistic Corrosion Inhibition Performance-A Comparative Analysis. 2024 - researchsquare.com
19. N. Raman, Phytochemicals techniques, New India Publishing Agency, New Delhi, pp. 19-25 (2006).
20. M.P. Madan, G. Raghavan, K.S.R. Ajay and P. Prabhu, Acta Pharm., 55, 297 (2005).
21. M.S. Gião, M.L. González-Sanjosé, M.D. Rivero-Pérez, C.I. Pereira, M.E. Pintado and F.X. Malcata, J. Sci. Food Agric., 87, 2638 (2007); https://doi.org/10.1002/jsfa.3023
22. Thirumurugan, A., Padmanaban, M., Kumar, D. S., Govindharaju, R., Padmavathy, S., Karthikeyan, V., ... & Karthik, S. (2026). Phytochemical and antioxidant properties of Kedrostis foetidissima and its larvicidal potential against mosquitoes. South African Journal of Botany.
23. Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods, 65, 55-63.
24. Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J., Boyd, 1991. Feasibility of high flux anticancer drug screen using a diverse panel of cultured human tumour cell lines. Journal of the National Cancer Institute, 83, 757-766.
25. Meenaloshini, P., Thirumurugan, A., Mohan, P.K. and Kumar, T.S., 2024. In Vitro Anticancer Efficacy of Hydroalcoholic Extract From Crateva religiosa G. Forst. Bark on Human Ovarian Cancer Cells. Journal of Herbal Medicine, 45, p.100870.

26. G.Jeevanantham,T. Aravindhan, G. Subashini, K. Priya , J. Gowri, P.Anitha, J. Nasrin Begum, D.Sathish Kumar, A.Thirumurugan. (2025). Fabrication And Functional Evaluation Of Copper Oxide Nanoparticles Utilizing Cardiospermum HalicacabumL. Leaf Extract Via Biogenesis. Journal of Applied Bioanalysis, 11(9s),124-131. https://doi.org/10.53555/jab.v11si9.1219
27. A. Saleem, I. Naureen, M. Naeem, G. Tasleem, H. Ahmed, and U. Farooq, "Therapeutic Role of Piper nigrum L (Black Pepper) and Pharmacological Activities," Sch. Int. J. Biochem., vol. 5, no. 1, pp. 15–21 (2022).
28. D. Kumar, S. Kumar, and A. Kumar, "Extraction and characterization of secondary metabolites produced by bacteria isolated from industrial wastewater," J. Water Process Eng., vol. 40, no. November, p. 101811 (2021)
29. Sakthivel V, Ilanthiraya S, Jeevanantham G, Aravindhan T, Priya K, Thirumurugan A, Larvicidal Activity Of Marine Macroalgae: A Comprehensive Review. Int J Drug Deliv Technol. 2026;16(4s): 908-917; DOI: 10.25258/ijddt.16.4s.106
30. Sandhiya T, Jeyabharathi S, Thirumurugan A. Exploration of Antimicrobial Drug Targets from Human Breast Milk Lactobacillus for the Control of Vaginal Infections. Int J Drug Deliv Technol. 2026;16(7s): 924- 933; DOI: 10.25258/ijddt.16.7s.98.
31. R. Sharma, N. Kumari, M. . Ashawat, and C. P. . Verma, " Standardization and phytochemical screening analysis for herbal extracts: Zingiber officinalis, Rosc., Curcuma longa Linn., Cinnamonum zeylanicum Nees., Piper longum, Linn., Boerhaavia diffussa Linn. ," Asian J. Pharm. Technol., vol. 10, no. 3, p. 127 (2020).
32. K. Jeena, V. B. Liju, N. P. Umadevi, and R. Kuttan, "Antioxidant, Anti-inflammatory and Antinociceptive Properties of Black Pepper Essential Oil (Piper nigrum Linn)," J. Essent. Oil-Bearing Plants, vol. 17, no. 1, pp. 1–12 (2014).
33. K. Nagaraj, "Nanoparticle and Gene Therapy Strategies for Site-Specific Pain Management: A Review." Adv. Therap.8, no. 12 (2025): e00356. https://doi.org/10.1002/adtp.202500356.
34. Y. Gao et al., "Structure of RNA-dependent RNA polymerase from 2019-nCoV, a major antiviral drug target," bioRxiv, p. 2020.03.16.993386, 2020.
35. Jeyaram, M., Jeyaram, Y., Ayyadurai, T., Gurusamy, M. (2026). Exploration of Streptomycetes as Biocontrol Agents Against Plant Pathogens. In: Kaur, T., Kumar, H., Manhas, R.K. (eds) Streptomycetes: Biological Candidates for Sustainable Agriculture. Springer, Singapore. https://doi.org/10.1007/978-981-95-5803-2_6
36. J. P. Vincken, L. Heng, A. de Groot, and H. Gruppen, "Saponins, classification and occurrence in the plant kingdom," Phytochemistry, vol. 68, no. 3, pp. 275–297 (2007).
37. U. Bildziukevich, M. Wimmerová, and Z. Wimmer, "Saponins of Selected Triterpenoids as Potential Therapeutic Agents: A Review," Pharmaceuticals, vol. 16, no. 3 (2023).
38. S. Chitti et al., "Design, synthesis and biological evaluation of 7–(5–((substituted – amino)-methyl)-thiophen–2–yl)-spiro-[chroman–2,4′–piperidin]–4–one hydrochloride analogues as anticancer agents," Bioorg. Chem., vol. 112, no. January, p. 104865 (2021).
39. R. Chauhan, E. Chaudhary, and N. Chauhan, "Investigation of Inhibitory Activity and Bioactive Compounds of Piper nigrum Seeds on Selected Human Pathogens," Int. J. Curr. Microbiol. Appl. Sci., vol. 6, no. 7, pp. 2670–2679 (2017).
40. S. Kumar et al., "Anti-Inflammatory and Antioxidant Properties of Piper Species: A Perspective from Screening to Molecular Mechanisms," Curr. Top. Med. Chem., vol. 15, no. 9, pp. 886–893 (2015).
41. A. Rohman et al., "Comprehensive Review on Application of FTIR Spectroscopy Coupled with Chemometrics for," Molecules, vol. 25, no. 5485, pp. 1–28 (2020).
42. S. Sethi, A. Joshi, B. Arora, A. Bhowmik, R. R. Sharma, and P. Kumar, "Significance of FRAP, DPPH, and CUPRAC assays for antioxidant activity determination in apple fruit extracts," Eur. Food Res. Technol., vol. 246, no. 3, pp. 591–598 (2020).
43. Ayyadurai, T., Thiruppathi, S.K. and Diana, R.K., 2021. B. Effect of aqueous seed extract of Caesalpinia bonduc (L.) Roxb. on hormonal assay and lipid profile in induced polycystic ovary syndrome albino female rats. Int J Bot Stud, 6(3), pp.139-146.
44. Thirumurugan, A., Kumar, T.S. and Kumari, B.R., 2020. Influence of plant growth regulators on plant regeneration from epicotyl and hypocotyl explants of Caesalpinia bonduc (L.) Roxb–an ethnomedical plant. Journal of Applied Horticulture, 22(1), pp.80-84.
45. V. Chaudhary, R. RK, N. Chaudhary, and G. Sharma, "Bio-Control of Multiple Drug-Resistant Uropathogens Using Medicinal Plant Extracts," Asian J. Pharm. Clin. Res., vol. 12, no. 2, pp. 371–376 (2019)
46. R. M. Perez Gutierrez, "Antidiabetic andantioxidant properties, and α-amylase and α-glucosidase inhibition effects of triterpene saponins from Piper auritum," Food Sci. Biotechnol., vol. 25, no. 1, pp. 229–239 (2016).
47. S. Nandakumar, M. G. S. Kumar, B. Bini, and G. G. Krishnan, "Antimicrobial activity of selected medicinal plants against oral microflora," Res. J. Pharm. Technol., vol. 9, no. 12, p. 2271 (2016).
48. Bishayee, A., Ahmed, S., Brankov, N. and Perloff, M. (2011) Triterpenoids as Potential Agents for the Chemoprevention and Therapy of Breast Cancer. Frontiers in Bioscience: A Journal and Virtual Library, 16, 980-996. https://doi.org/10.2741/3730
49. Bommareddy, A., Eggleston, W., Prelewicz, S., Antal, A., Witczak, Z., McCune, D.F. and Vanwert, A.L. (2013) Chemoprevention of Prostate Cancer by Major Dietary Phytochemicals. Anticancer Research, 33, 4163-4174.
50. Kim, M.O., Moon, D-O., Choi, Y.H., Lee, J.-D., et al. (2008) Platycodin D Induces Mitotic Arrest in Vitro, Leading to Endoreduplication, Inhibition of Proliferation and Apoptosis in Leukemia Cells. International Journal of Cancer, 122, 2674-2681. https://doi.org/10.1002/ijc.23442

51. Kanchana, A. and Balakrishna, M. (2011) Anti-Cancer Effect of Saponins Isolated from Solanum trilobatum Leaf Extract and Induction of Apoptosis in Human La rynx Cancer Cell Lines. International Journal of Pharmacy and Pharmaceutical Sciences, 3, 356-364.
52. Lu, C., Li, C.J., Wu, D., Lu, J.M., Wang, L. and Tu, F. (2011) Induction of Apoptosis by Rhizoma Paridis Saponins in MCF-7 Human Breast Cancer Cells. African Jour nal of Pharmacy and Pharmacology, 5, 1086-1091.
53. Azam, J. Y. Park, I. S. Kim, and D. K. Choi, "Piperine and ItsMetabolite's Pharmacology in Neurodegenerative and Neurological Diseases," Biomedicines, vol. 10, no. 1, pp. 1–16 (2022).
54. M. R. M. de Brito et al., "Cyclohexene-fused 1,3-oxazines with selective antibacterial and antiparasitic action and low cytotoxic effects," Toxicol. Vitr., vol. 44, pp. 273–279 (2017)
55. Z. S. Al-Dabbagh, "The Role of Decision-maker in Crisis Management: A qualitative Study Using Grounded Theory (COVID-19 Pandemic Crisis as A Model)," J. Public Aff., vol. 20, no. 4 (2020).
56. A. M. Pisoschi, A. Pop, F. Iordache, L. Stanca, G. Predoi, and A. I. Serban, "Oxidative stress mitigation by antioxidants - An overview on their chemistry and influences on health status," Eur. J. Med. Chem., vol. 209, p. 112891 (2021).
57. H. A. Ahmed and I. Y. Alkali, "In silico molecular docking studies of some phytochemicals against peroxisome-proliferator activated receptor gamma (PPAR-γ)," GSC Biol. Pharm. Sci., vol. 5, no. 2, pp. 001–005 (2018).