Journal of Advanced Zoology

ISSN: 0253-7214

*Volume **45** Issue **6** Year **2024** Page **01-20***

"Unveiling Phoenix sylvestris: Phytochemical Insights, Antioxidant Potential and Antiproliferative Impact on HT29 Cells"

Phillips Shamroy R[1], Padmini R[2]*

*[1,2]*Department of Biochemistry, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai-600117, Tamil Nadu, India*

***Corresponding Author:** Padmini R*

**Department of Biochemistry, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai-600117, Tamil Nadu, India, Email: padmini.sls@velsuniv.ac.in, Phone: 9941165319*

Abstract

Phoenix sylvestris, commonly known as the wild date palm, holds immense potential in traditional medicine due to its diverse phytochemical composition. Leaf identification plays a crucial role in botanical research, ecological studies, and biodiversity conservation. *Phoenix sylvestris*, commonly known as the wild date palm or silver date palm, is an economically and ecologically significant palm species. Traditional methods of identifying plant species based on morphological characteristics can be challenging, especially when dealing with closely related species or in cases of morphological variation. In this study, we present a DNA-based approach for the identification of *Phoenix sylvestris* leaves through DNA extraction, amplification of specific genetic markers, and sequencing. Leaf samples were collected from various geographical locations to encompass genetic diversity. DNA was extracted using a modified CTAB method, and the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA was amplified using polymerase chain reaction (PCR). Sanger sequencing was performed to obtain DNA sequences, which were then compared to reference sequences in public databases for species identification. The results demonstrate the efficacy of this approach in accurately identifying *Phoenix sylvestris* leaves, even in cases where morphological characteristics may be ambiguous or variable. This DNA-based method provides a reliable tool for rapid and accurate identification of *Phoenix sylvestris*, contributing to its conservation and management efforts, as well as facilitating research on its ecology, distribution, and evolutionary history. This study presents a multifaceted analysis of *P. sylvestris* extract, encompassing phytochemical characterization, antioxidant, antimicrobial, and anticancer evaluations, along with molecular docking simulations. The phytochemical analysis revealed the presence of various bioactive compounds such as phenolics and flavonoids contributing to its medicinal properties. Assessment of antioxidant activity through DPPH assay demonstrated significant radical scavenging potential, indicating its potential therapeutic application in oxidative stress-related disorders. Moreover, the antimicrobial evaluation against a panel of pathogenic microorganisms highlighted the extract's efficacy in inhibiting microbial growth, suggesting its utility as a natural antimicrobial agent. Furthermore, the anticancer potential was evaluated against various cancer cell lines, revealing promising cytotoxic effects, particularly against specific cancer types. Molecular docking studies provided insights into the interaction between bioactive compounds

of *P. sylvestris* extract and key molecular targets implicated in cancer progression, validating its potential as a source of novel anticancer agents. Overall, this comprehensive investigation underscores the pharmacological significance of *P. sylvestris* extract, emphasizing its therapeutic versatility and potential for drug discovery and development.

Keywords: ***Colon cancer, Molecular docking, antioxidants, Phoenix sylvestris, Medicinal plants.***

Introduction

Colorectal cancer, often referred to as bowel cancer, is a malignancy that affects the colon or rectum [1], which is the third most frequent cancer in the world. It ranks second among cancers in women and third among cancers in males. It typically begins as a growth of tissue known as a polyp, which can be benign or cancerous. Over time, cancerous polyps can grow and invade nearby tissues or spread to other parts of the body, leading to serious health complications.

It affects both men and women, usually occurring in individuals over the age of 50, although it can develop at any age. While the exact causes of colorectal cancer are not fully understood, factors such as age, family history, certain genetic mutations, diet high in red or processed meats, low-fiber and high-fat diet, sedentary lifestyle, obesity, smoking, and heavy alcohol consumption are known to increase the risk [2]. In 2020, there were about 1.9 million brand-new cases of colorectal cancer and more than 930,000 deaths from the disease. About 153,020 people were diagnosed with CRC in 2023. Large and small bowel cancer incidence rates are low in India, and rectal cancer is more prevalent than colon cancer. In eight population registries, the incidence rates of colon cancer range from 3.7 to 0.7/100,000 for men and 3 to 0.4/100,000 for women. The incidence rates for rectal cancer range from 5.5 to 1.6/100,000 for men and from 2.8 to 0/100,000 for women. In India, the incidence of large bowel cancer is almost half that in metropolitan areas [3]. Early detection and treatment are crucial for improving outcomes and survival rates. Screening tests such as colonoscopies can detect precancerous polyps or early-stage cancer when it's most treatable. Treatment options for colorectal cancer include surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy, depending on the stage and location of the cancer, as well as the overall health of the patient [4].

Medicinal plants harbour bioactive compounds like flavonoids, alkaloids, and polyphenols, renowned for their robust anti-cancer attributes, capable of pinpointing cancer cells while preserving healthy tissue integrity, thus mitigating adverse effects. Moreover, these plants boast rich traditional usage across diverse cultures, offering a repository of empirical wisdom that serves as a valuable compass for contemporary scientific exploration.

Indian dates, recognized scientifically as *Phoenix sylvestris*, originate from the southern regions of Pakistan and India, carrying historical significance and global renown for their nutritional richness. Commonly referred to as the "Wild date palm," this traditional plant also goes by synonymous names like Date-sugar palm, Indian wild date, Indian wine palm, Silver date palm, Sugar date palm, and Sugar palm. This fruit boasts a spectrum of essential nutrients including carbohydrates, phenols, amino acids, flavonoids, tannins, alkaloids, terpenoids, dietary fiber, and vital vitamins and minerals. Notably, different parts of the plant offer distinct medicinal attributes such as antipyretic, cardiotonic, laxative, diuretic, and antioxidant properties [5]. According to Kothari (2011), the seeds of the plant have been noted for their bacteriostatic effects against both Gram-positive and Gram-negative organisms. Additionally, traditional medicinal practices, as cited by Beg & Singh (2015) and Ghani (1998), utilize these seeds in treating conditions such as dysentery, ague, and diabetes. Lima *et al*. (2010) conducted an assessment of the crude ethanolic extract from the leaves of *P. paludosa* for its antidiarrheal effects using a mouse model of castor oil-induced diarrhea. Their findings indicated a dose-dependent response, with the extract significantly reducing the frequency of liquid stools in rats. The purified methanol fractions from the leaf extracts of *P. sylvestris* inhibited the cell growth in J774 and THP1 cell lines (sharma *et al*, 2016). These fractions demonstrated enhanced antioxidant and antibacterial properties, as well as cytotoxic effects. Importantly, compared to the crude extract, the purified fractions notably induced reactive oxygen species (ROS) generation [6]. Despite the variety of pharmacological uses, the plant has not been explored for study against colon cancers. Therefore, the present study is intended to ascertain the anticancer effect of *Phoenix sylvestris* through *insilico* and invitro analysis.

The pursuit of enhanced DNA extraction methods has spurred the creation of diverse procedures, although the core principles of DNA extraction remain consistent. Preserving biological material purity is vital to deter DNA degradation. As a result, even rudimentary extraction techniques can provide ample DNA suitable for a multitude of applications. Various materials can be employed to extract DNA from plant tissue depending on

the medium [7]. Any mechanical method capable of breaking down cell walls and membranes without causing damage can access the nuclear material. Typically, this involves grinding the cell wall material with liquid nitrogen, which deactivates potentially harmful cellular enzymes and chemicals while facilitating DNA access. Once adequately pulverized, the tissue can be suspended in a suitable buffer, such as CTAB. Centrifugation is then utilized to eliminate insoluble particles from the DNA, while a combination of chloroform and centrifugation is employed to separate soluble proteins and other substances. Subsequently, DNA is precipitated from the aqueous phase and thoroughly washed to eliminate any contaminating salts. The purified DNA is then suspended and preserved using TE buffer or sterile distilled water. This method has been validated to yield intact genomic DNA from plant tissue. To evaluate the purity of the extracted DNA, a sample is subjected to electrophoresis on an agarose gel, stained with ethidium bromide, and examined under a UV lamp [8].

MATERIALS AND METHODS

Collection of Sample:

The leaves parts of *Phoenix sylvestris* was purchased from Annai Violet college of Arts and Science Ambattur, Chennai - 600 053, Tamil Nadu. The plants were authenticated by Professor P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), West Tambaram, Chennai, Tamil Nadu. The collected samples were transported to the laboratory immediately. The leaves collected were washed with sterile distilled water to remove any adherent particles and blotted to remove excess water. The samples were cut into small pieces and shade dried for five days of time. After drying, the samples were grounded and stored for further analysis.

Extraction:

Clean palm leaves (*Phoenix sylvestris*) were taken and washed thoroughly in a clean tap water and dried it under the sun shade, at room temperature for two weeks. The leaf powder was macerated in methanol for 3 days. On the fourth day using rotary evaporator gentle removal of solvents was done by evaporating under 130 – 150 rpm and 45 – 60ºC. The extract derived was used for further analysis.

Identification & Isolation of DNA Extraction

Plant Genomic DNA Extraction using CTAB

Grind 200 milligrams of plant tissue into a fine paste using approximately 500 microliters of CTAB buffer. Transfer the CTAB/plant extract mixture to a microfuge tube and incubate it in a recirculating water bath at 55°C for approximately 15 minutes. Following incubation, centrifuge the CTAB/plant extract mixture for 5 minutes at 12,000 g to precipitate the cell debris. Carefully transfer the supernatant into sanitized microfuge tubes [9].

Add 250 microliters of Chloroform: Isoamyl Alcohol (24:1) to each tube and gently invert the tubes to mix the contents thoroughly. Following mixing, centrifuge the tubes for one minute at 13,000 rpm to facilitate phase separation. Carefully transfer only the upper aqueous phase, containing the DNA, to a sterile microfuge tube.

To precipitate the DNA, add 50 microliters of 7.5 M ammonium acetate and 500 microliters of ice-cold 100% ethanol to each tube. Slowly invert the tubes multiple times to ensure even mixing and promote DNA precipitation. Alternatively, allow the tubes to sit at -20°C for one hour after adding ethanol to enhance DNA precipitation.

After precipitation, remove the DNA using a pipette, gently spinning or rotating the tip in the cooled solution. The precipitated DNA will form a thick, transparent precipitate adhering to the pipette tip.

For DNA washing, transfer the precipitate into a microfuge tube containing 500 microliters of ice-cold 70% ethanol and slowly invert the tube. Repeat this washing step. Alternatively, isolate the precipitate by centrifuging the tube at 13,000 rpm for one minute to form a pellet. Discard the supernatant and wash the DNA pellet by adding two changes of ice-cold 70% ethanol.

Following washing, centrifuge the DNA pellet at 13,000 rpm for 1 minute to consolidate the pellet. Remove all the supernatant and allow the DNA pellet to air-dry for approximately 15 minutes. Be cautious not to over-dry the DNA as it may become difficult to re-dissolve.

Re-suspend the DNA in sterile, DNase-free water (between 50 and 400 microliters H_2O); the volume of water needed for DNA dissolution varies depending on the isolation yield. To eliminate any residual RNA, add RNase A (10 µg/ml) to the water before DNA dissolution (10 µl RNase A in 10 ml H_2O). After resuspension, store the DNA at 4°C and incubate it for 20 minutes at 65°C to inactivate any potential DNases. Spectrophotometry can be utilized to determine the concentration and purity of the DNA, while agarose gel electrophoresis will confirm the integrity of the DNA.

DNA quality confirmation
Heat 1 gram of agarose in 100 millilitres of 0.5x TBE buffer in a microwave for approximately 2 minutes until it dissolves, resulting in a 1% agarose solution. After allowing the solution to cool for a brief period, add 2.5 microliters of ethidium bromide and stir thoroughly to ensure even distribution [9]. Proceed to cast the gel using the provided tray and comb, then allow the gel to solidify on a level surface for a minimum of 20 minutes at room temperature.

Load the following into separate wells
- 10 µL 1kb ladder
- 5 µL sample + 5 µL water + 2 µL 6x Loading Buffer

Operate the gel electrophoresis at 100 volts for a duration of 30 minutes. Following electrophoresis, position the gel beneath UV light and capture an image to illustrate the results. Evaluate the DNA quality: a distinct, high molecular weight band signifies intact, high-quality DNA, whereas a smeared band indicates DNA degradation [10].

Phytochemical Qualitative Analysis

Test for alkaloids
A tube containing 5 mg of Dragendorff test extract was used. Upon adding a single drop of Dragendorff's reagent, an orange-red precipitate formed, indicating the presence of alkaloids.

Test for carbohydrates
Fehling's test was performed by combining 5 mg of extract with a few drops of Benedict's reagent, followed by boiling. Carbohydrates were identified through the formation of a reddish-brown precipitate.

Tests for Glycosides
10 ml of aqueous plant extract and 1 ml of concentrated H2SO4 were mixed with a 4.0 ml solution of glacial acetic acid and a drop of 2.0% FeCl3 solution, a brown ring formed at the interface, indicating the presence of cardiac steroidal glycosides.

Test for Saponins
A test tube containing 5.0 ml of distilled water and aqueous crude plant extract was vigorously stirred, leading to the appearance of foam, indicating the presence of saponins.

Test for proteins
To determine the protein content, a few drops of biuret's reagent were added to 5 mg of the test extract. The resulting mixture was thoroughly mixed and warmed for 1 to 5 minutes. The presence of a red or violet colour indicated the presence of proteins.

Test for Amino acids
For the Ninhydrin test, 5 mg of sample extract was mixed with 2 ml of a 0.2% Ninhydrin solution, and the mixture was heated for 2 minutes using a water bath. The development of a violet colour indicated the presence of amino acids.

Test for phenols
When the powdered leaf sample is boiled and filtered in 20 ml of distilled water in a test tube, adding three to four drops of 0.1% ferric chloride to the filtered sample results in a colour change to brownish-green or blue, indicating the presence of phenols.

Test for Fixed oil
The extract was pressed between two filter sheets in a small quantity. The appearance of an oil stain on the paper indicated the presence of fixed oil.

Test for Terpenoids
Combining 5 ml of the aqueous plant extract with 2.0 ml of chloroform, followed by evaporation on a water bath and heating with 3 ml of concentrated H_2SO_4, resulted in the formation of terpenoids, indicated by a grey tint appearing.

Phytochemical Quantitative analysis
Determination of total phenolic content (Singleton and Rossi, 1965)

The plant powder (2g) underwent soaking in various solvents for 24 hours in methanol. After filtration of the residues, the filtrate was evaporated. The resulting plant extracts were then centrifuged for 15 minutes at 4°C at 10,000 rpm. The supernatant was utilized to prepare 20 mL of extract, which was mixed with 3 mL of distilled water. Subsequently, 0.5 mL of phenol reagent from Folin-Ciocalteu was added to each tube. The tubes were then incubated for 3 minutes at 45°C. After 3 minutes, 2 mL of 20% Na_2CO_3 was added to each tube, and the absorbance of each tube was measured at 650 nm [11]. Finally, the total phenol content in the sample was calculated using a specific formula,
C (GAE) = c x V/M
where, c = concentration of sample from the curve obtained (mg/mL),
V = volume used during the assay (mL) and M = mass of the sample used during the assay (g)

Determination of total flavonoids

Flavonoid contents were determined using a slightly modified version of Karadeniz *et al*. (2005)'s spectrophotometry approach. To begin, 1 g of dry powder was ground in a mortar and pestle using 200 mL of 80% aqueous methanol. The resulting mixture was filtered to produce a transparent filtrate. In a test tube, an aliquot of the sample (0.5 mL) was combined with 3 mL of distilled water and 0.3 mL of 5% sodium nitrite. After vortexing and allowing it to stand at room temperature for five minutes, 0.6 mL of 10% aluminium chloride was added to the solution. Following an additional 6 minutes, 2 mL of 1 M sodium hydroxide was introduced into the test tube [12].
The solution was made up to 10 mL with distilled water. The absorbance was read at 510 nm. The total flavonoid content was calculated as quercetin equivalent (mg QE/g) using the formula,
$X = (A\text{-}M_0/A_0\text{-}M)$
where, A= absorption of sample, A_0= absorption of standard (quercetin),
M= weight of sample (mg/mL) and M_0= weight of quercetin in solution (mg/mL)

Total Antioxidant assay

In each test tube, 1 mL of distilled water, 1 mL of molybdate reagent solution, 1 mL of sodium phosphate, and 1 mL of sulfuric acid were added separately. The extract was then added at various concentrations ranging from 10 to 100 µg/ml. These tubes were incubated at 95°C for 90 minutes. Following incubation, the tubes were allowed to cool to room temperature for 20–30 minutes. The absorbance of the reaction mixture was then measured at 695 nm, and the values were recorded [13].

DPPH assay

The percentage of antioxidant activity for each substance was determined using the DPPH free radical assay, following the protocol described by Szabo *et al*. (2007). The assay involved utilizing the stable DPPH radical to react with the samples in a methanol solution. Specifically, 0.5 mL of sample, 1 mL of methanol, and 1 mL of a 0.5 mM DPPH radical solution were added to the reaction mixture. The reduction of DPPH occurs when it interacts with an antioxidant substance capable of donating hydrogen [14].
The colour changes (from deep violet to light yellow) were assessed by measuring the absorbance (Abs) at 517 nm after 100 minutes of reaction using a UV-VIS spectrophotometer. A control solution was prepared by mixing 1.0 mL of methanol with 1.0 mL of the DPPH radical solution, while 1 mL of methanol served as a blank. The scavenging activity percentage was determined according to the following formula:
% of inhibition = Control O.D – Sample O.D/Control O.D X 100.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is the predominant analytical technique utilized for nerolidol detection, primarily due to the favourable boiling points of sesquiterpenes, which typically fall within the range suitable for gas-phase separation techniques employed by GC-MS analysis (~250 to 280 °C). In addition to GC-MS, liquid chromatography-mass spectrometry (LC-MS) is also widely employed, offering notable advantages such as heightened sensitivity and accuracy. These findings suggest that GC-MS might be

preferred for nerolidol detection [15]. To isolate nerolidol from an antioxidant DPPH assay of *Phoenix sylvestris* methanol extract using GC-MS, begin by preparing the methanol extract through a suitable extraction method. Perform the DPPH assay to evaluate antioxidant activity, then proceed to isolate nerolidol from the methanol extract. Employ chromatographic techniques like column chromatography or preparative thin-layer chromatography (TLC) to separate nerolidol from other constituents. After collecting nerolidol-containing fractions, concentrate them and dissolve in a suitable solvent for GC-MS analysis. Inject the sample into the GC-MS system equipped with a capillary column and analyse under appropriate conditions. Identify nerolidol by comparing retention time and mass spectrum with reference standards. Characterize and quantify nerolidol based on its GC-MS data. Analyse the relationship between nerolidol concentration and antioxidant activity measured in the DPPH assay. Validate results through statistical analysis and report the procedure, findings, and conclusions obtained from the isolation and analysis of nerolidol [16].

Anticancer Activity
Cell Lines

HT29 cell lines were purchased from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100µg/ml) in a humidified atmosphere of 50µg/ml CO2 at 37 °C [17]. HT29 cell lines were obtained from the National Centre for Cell Sciences, Pune (NCCS). These cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) under a humidified atmosphere with 5% CO_2 at 37°C.

MTT assay

The MTT test was employed to detect cytotoxicity, which is characterized by a loss of viable cells. This assay relies on the metabolic reduction of the soluble MTT salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, into an insoluble coloured formazan product, which is quantified spectrophotometrically (Sadeghi-aliabadi *et al*., 2010). It reflects the normal function of mitochondrial dehydrogenase activity and cell viability. The number of viable cells can be determined by assessing the activity of mitochondrial dehydrogenase in living cells (Mosmann, 1983).
In this assay, cells were exposed to the extract and then incubated for 72 hours at 37°C and 5% CO_2. Subsequently, each well received 20 µl of filter-sterilized MTT solution (2 mg/ml) in phosphate-buffered saline (PBS) and was incubated for 3 hours at 37°C. After removing the MTT medium and adding 100 µl of dimethyl sulfoxide (DMSO) to solubilize the formazan crystals, the absorbance was measured at 540 nm using a universal microplate reader [18]. A comparison between treated and untreated cells was conducted. Only metabolically active cells (viable cells) are capable of enzymatically reducing tetrazolium salts to formazan colour, thus providing insight into cell viability.

LDH assay

The LDH release assay is a method used to measure the amount of lactate dehydrogenase (LDH), an enzyme released from cells when they are damaged or undergoing cell death. In the context of HT29 cell lines, which are commonly used in cancer research, LDH release serves as a surrogate marker for cell death [19]. The assay involves transferring a 50 µl sample from a Sample Plate to an Assay Plate. Then, 50 µl of Assay Buffer is added, followed by a 1-hour incubation period. After incubation, 50 µl of Stop Solution is added, and the absorbance of the solution is measured at a wavelength of 490 nm. This workflow provides a fast and reliable method for quantifying normalized LDH release, which can be indicative of cellular damage or death in HT29 cell lines.

***In silico* analysis**

The Argus lab tool was employed to carry out receptor-ligand docking, with preparation of both the ligand and protein accomplished through the Avogadro program.

Selection of target protein and Preparation of protein target structure:

In this study, the target protein receptors utilized for the docking procedure were sourced from the RCSB Protein Data Bank database. Input files were prepared using the Argus lab program, with all water molecules,

random residues, and ions completely removed from the protein receptors to ensure accuracy. To optimize the docking process, hydrogen atoms were also incorporated into the receptors.

Selection and preparation of Ligand molecule:

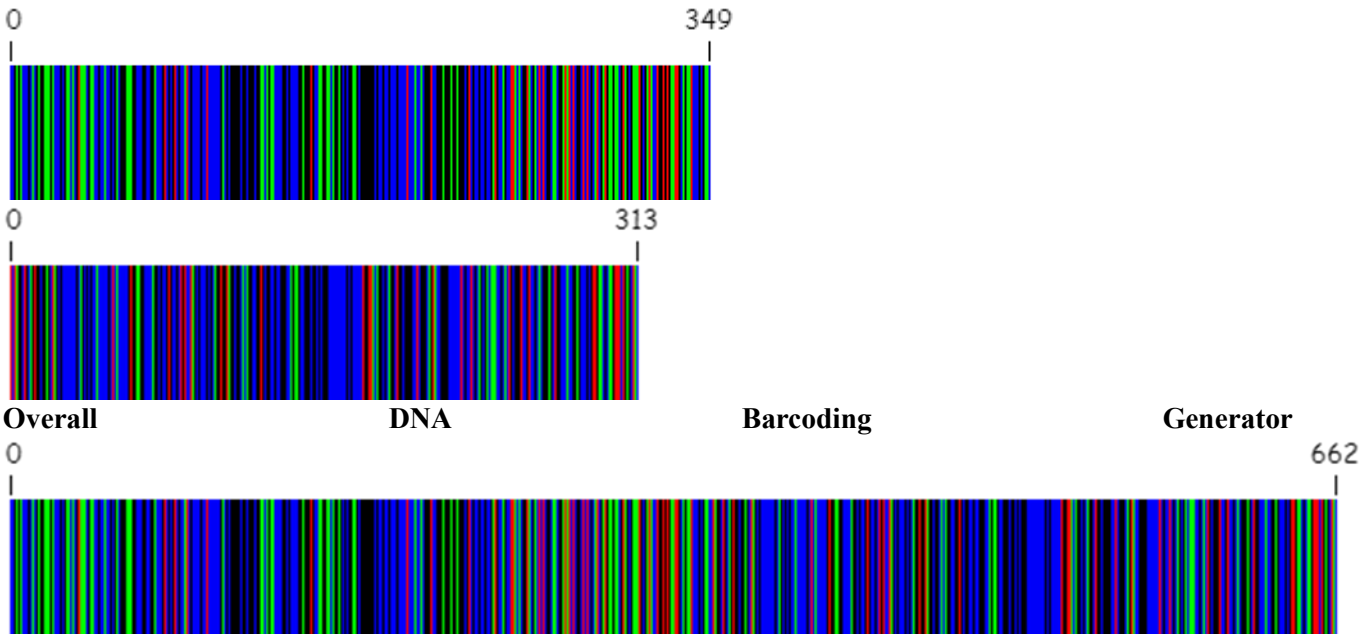
Avogadro, an advanced semantic chemical editor, visualization, and analysis platform, was employed to ascertain the 3D structure of nerolidol. The 'correction' feature was utilized to adjust the torsion tree, non-polar hydrogens, charges, and atom types after the three-dimensional structure was imported into the Argus lab software.

Molecular docking investigation:

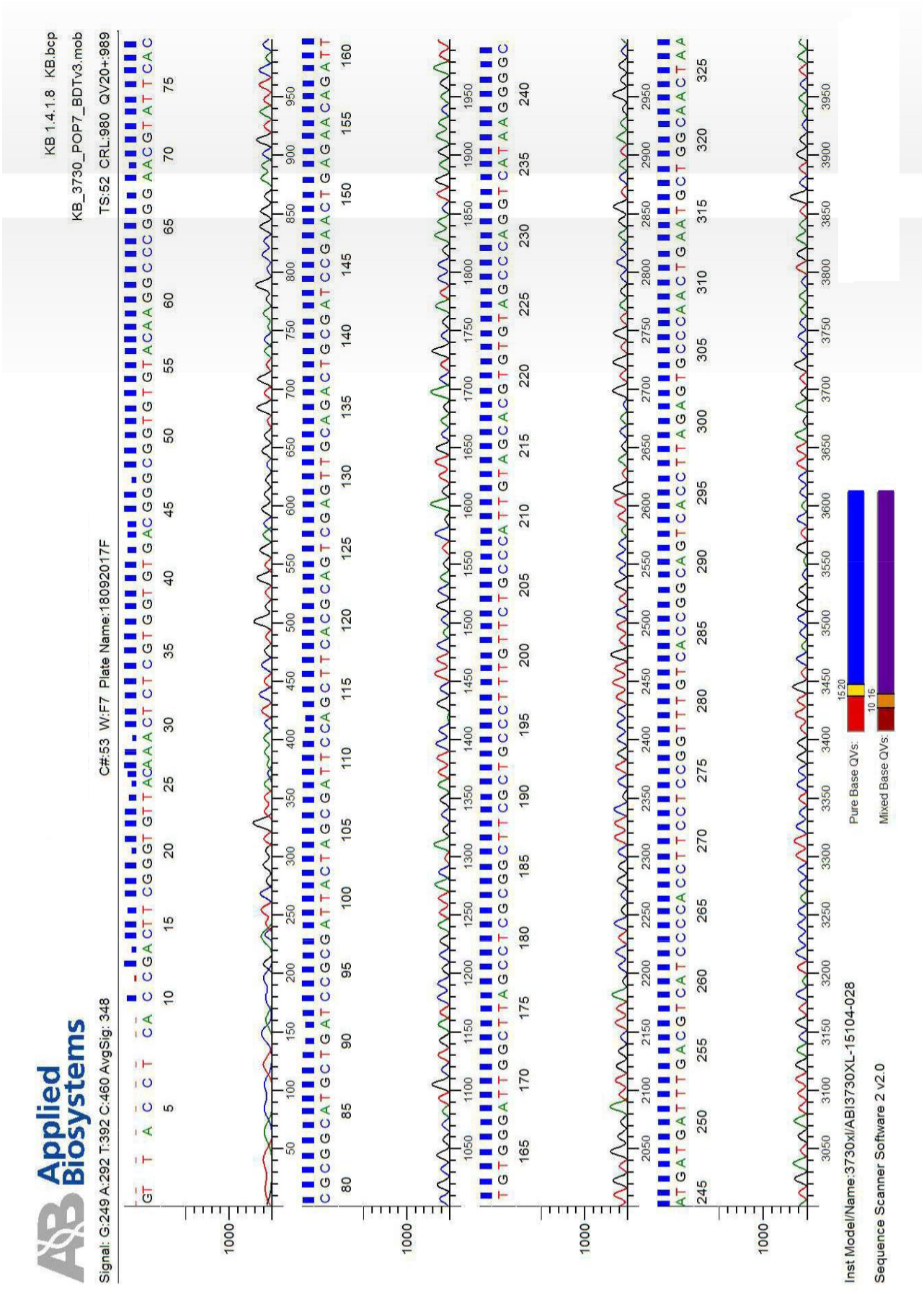
Molecular docking analysis was conducted with several potential targets, namely between GSK 3, AXIN, Porcine Osteocalcin, DVL, and E- Cadherin. Initially, the protein structure was uploaded as a macromolecule, followed by the elimination of water molecules and random residues. Subsequently, the selected bioactive substance, nerolidol (ligand), was uploaded after the amino acids were selected [20]. Water molecules were removed, and residues were designated as the ligand site. The software then minimized the ligand's energy and transformed it into Auto dock ligand format (pdbqt). Blind docking commenced after covering the entire protein structure under the grid box to screen the best-fit bioactive chemicals based on energy values. Each simulation consisted of five iterations, resulting in five docked conformations. It was concluded that the configurations with the lowest energy were the ones with the highest binding affinities [21]. The intra-molecular interactions, including hydrogen bonds, van der Waals forces, and hydrophobic interactions, were thoroughly analysed using Argus lab.

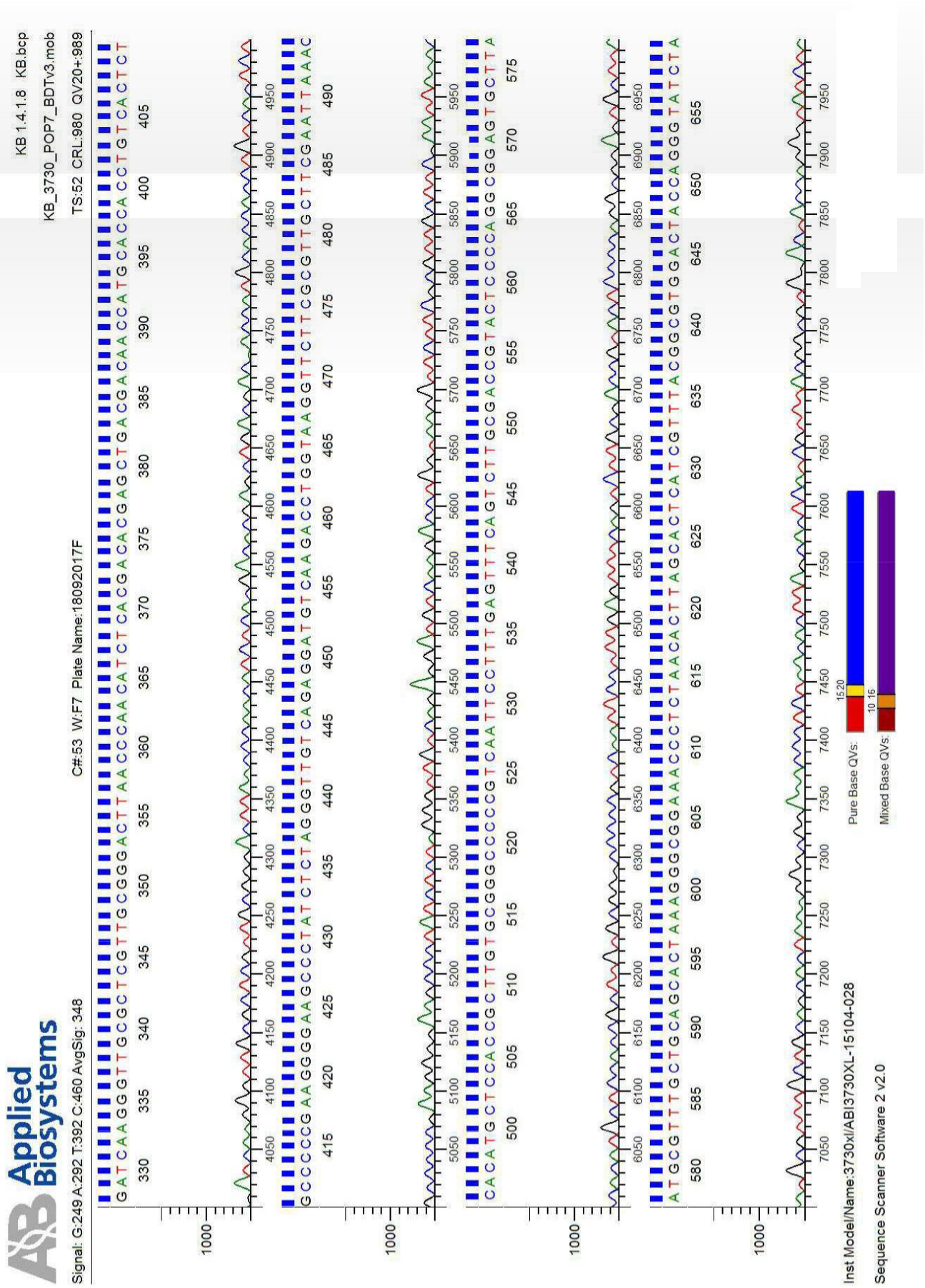
Results

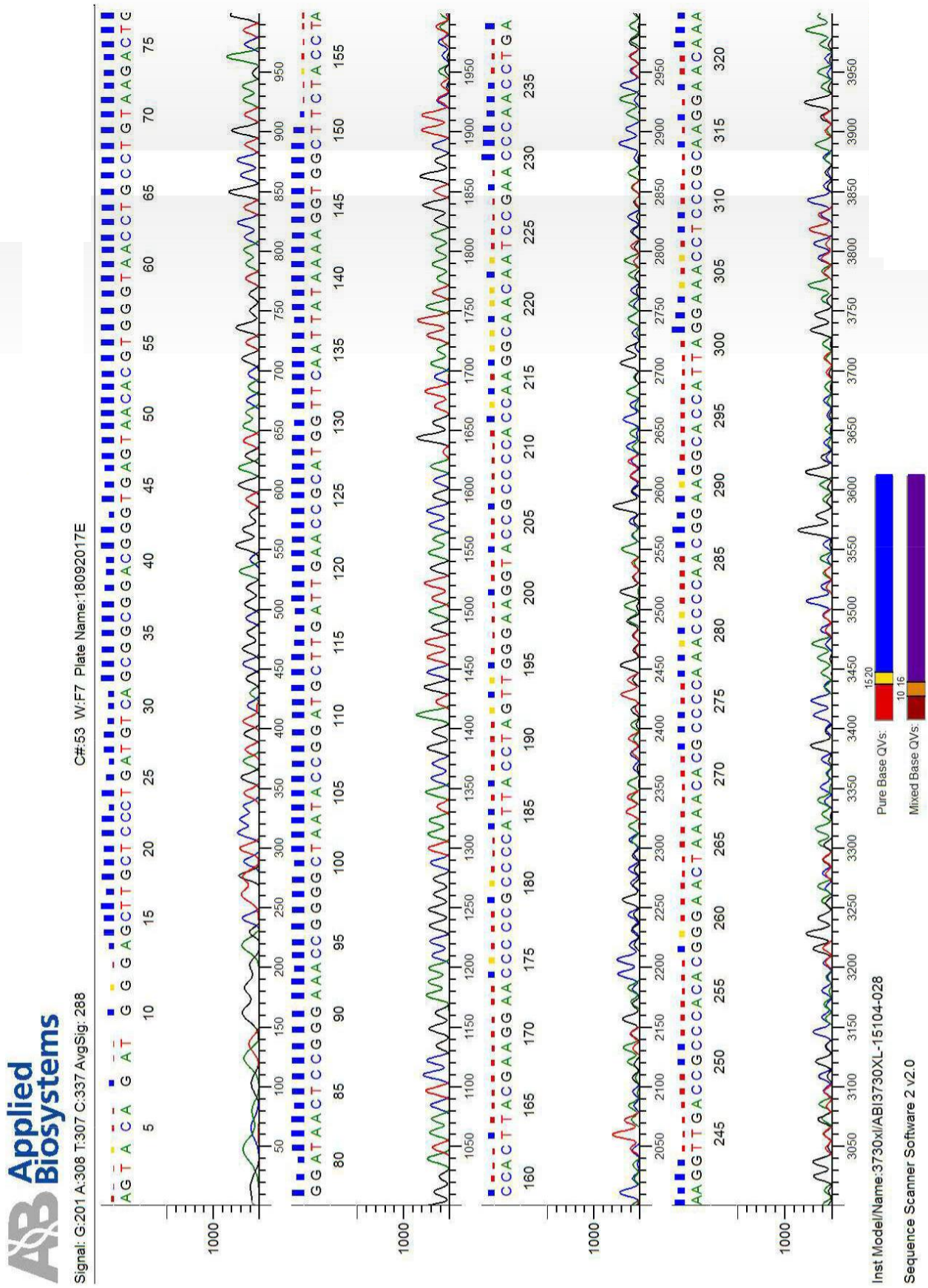
Identification & Isolation of DNA Extraction
***Phoenix sylvestris* DNA Barcoding Generator**

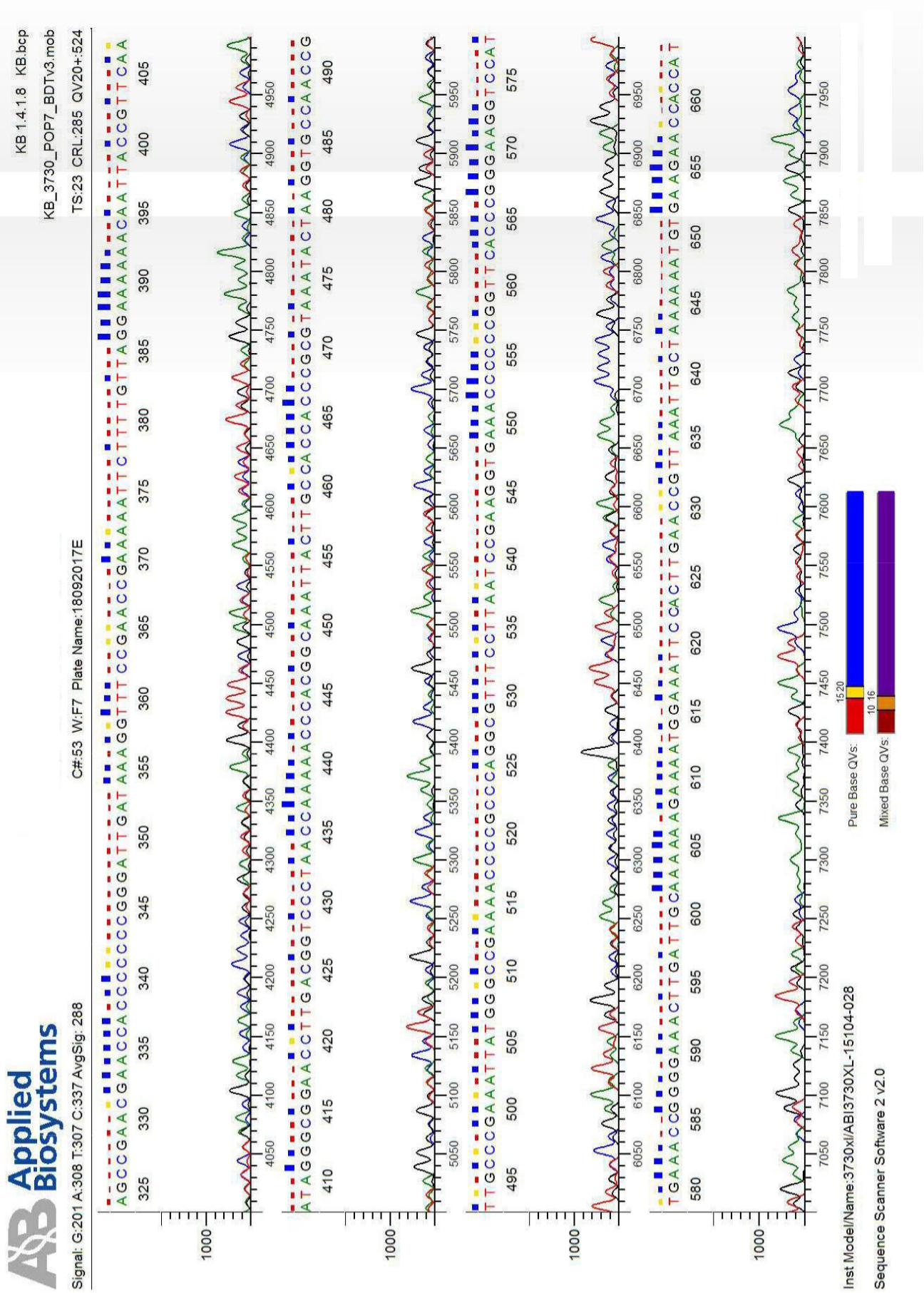


The DNA barcode generator allows you to create a barcode in color — each line of the barcode represents a particular base pair: **Green = Adenine, Blue = Cytosine, Black = Guanine, Red = thymine.** When assembled side by side, you will create a representative color barcode for the sequence of your sample!


Applied Biosystems
KB 1.4.1.8 KB.bcp
KB_3730_POP7_BDTv3.mob
Signal: G:249 A:292 T:392 C:460 AvgSig: 348
C#:53 W:F7 Plate Name:18092017F
TS:52 CRL:980 QV20+:989
Pure Base QVs:
Mixed Base QVs:
Inst Model/Name:3730xl/ABI3730XL-15104-028
Sequence Scanner Software 2 v2.0


Applied Biosystems
KB 1.4.1.8 KB.bcp
KB_3730_POP7_BDTv3.mob
Signal: G:249 A:292 T:392 C:460 AvgSig: 348
C#:53 W:F7 Plate Name:18092017F
TS:52 CRL:980 QV20+:989
Inst Model/Name:3730xl/ABI3730XL-15104-028
Pure Base QVs:
Mixed Base QVs:
Sequence Scanner Software 2 v2.0


Applied Biosystems
Signal: G:201 A:308 T:307 C:337 AvgSig: 288
C#:53 W:F7 Plate Name:18092017E
Pure Base QVs:
Mixed Base QVs:
Inst Model/Name:3730xl/ABI3730XL-15104-028
Sequence Scanner Software 2 v2.0


Applied Biosystems
KB 1.4.1.8 KB.bcp
KB_3730_POP7_BDTv3.mob
Signal: G:201 A:308 T:307 C:337 AvgSig: 288
C#:53 W:F7 Plate Name:18092017E
TS:23 CRL:285 QV20+:524
Pure Base QVs:
Mixed Base QVs:
Inst Model/Name:3730xl/ABI3730XL-15104-028
Sequence Scanner Software 2 v2.0

Phytochemical Qualitative Analysis

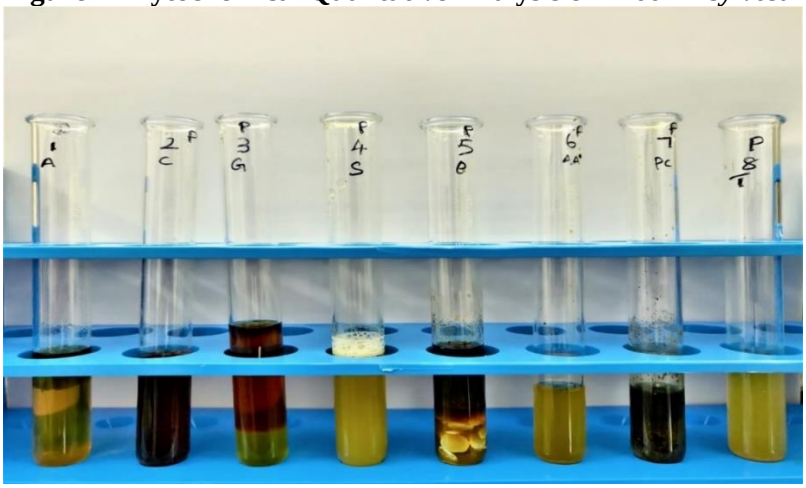
The current investigation focused on examining the methanol extract of *Phoenix sylvestris* to identify medically significant phytochemicals. The analysis revealed the presence of several phytochemicals, including alkaloids, carbohydrates, saponins, proteins, amino acids, phenolic compounds, and fixed oil. Notably, glycosides and terpenoids were found to be absent in the extract Shown in the Table 1 and Figure 1.

Table 1 Phytochemical Qualitative Analysis of *Phoenix sylvestris*

SI.NO	Test	Result
1	Alkaloids Test	+
2	Carbohydrates Test	+
3	Glycosides Test	−
4	Saponins Test	+
5	Proteins Test	+
6	Amino Acid Test	+
7	Phenolic Compounds Test	+
8	Fixed oil Test	+
9	Terpenoids Test	−

(+) = Positive; (−) = Negative

Figure 1 Phytochemical Qualitative Analysis of *Phoenix sylvestris*



Phytochemical Quantitative analysis

- The total phenol content in the methanol extract of *Phoenix sylvestris* was found to be 450 mg/g, equivalent to gallic acid as the standard.
- The total flavonoid content in the methanol extract of *Phoenix sylvestris* was determined to be 989 mg/g, equivalent to quercetin as the standard.
- The total protein content in the methanol extract of *Phoenix sylvestris* was measured at 28 µg/ml, equivalent to standard bovine serum albumin.
- Total crude lipid content in methanol extract of *Phoenix sylvestris* is 0.06%.
- The total carbohydrate content in the methanol extract of *Phoenix sylvestris* was determined to be 83 µg/ml, equivalent to standard D-glucose, Shown in the Table 2.

Table 2 Phytochemical Quantitative analysis of *Phoenix sylvestris*

SI NO	Test Sample (Methanol extract of *Phoenix sylvestris*)	Result
1	Total Phenol Test	450 mg/g
2	Total Flavonoids Test	989 mg/g
3	Total Proteins Test	28 µg/ml
4	Total crude Lipid Test	0.06%
5	Total Carbohydrates Test	83 µg/ml

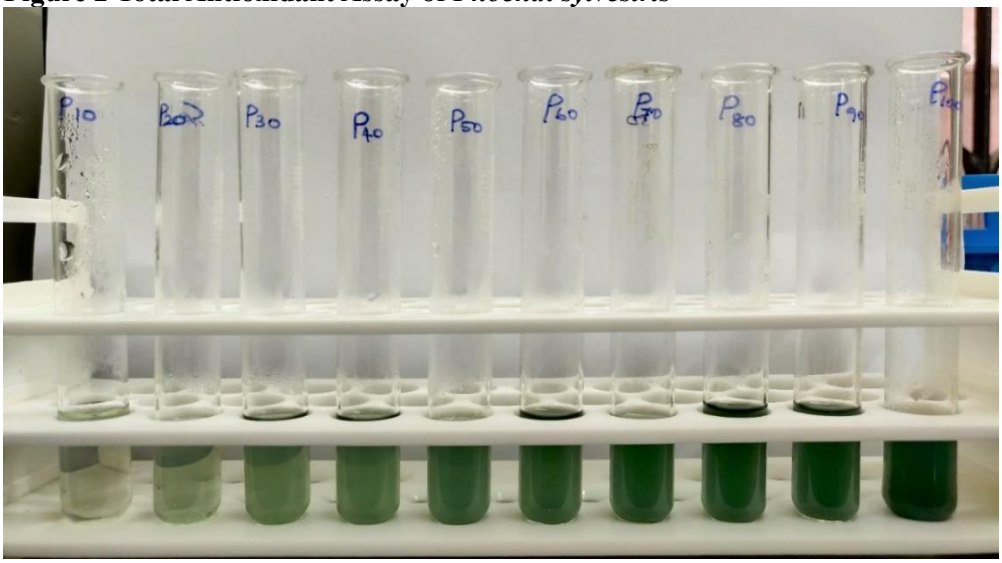
Antioxidant Activity
Total Antioxidant Assay

The green chromogen generated from the methanol extract of *Phoenix sylvestris* exhibited a maximum total antioxidant property with an absorbance of 1.861 at a concentration of 100 µg/ml Shown in the Table 3, Figure 2 & Graph 1.

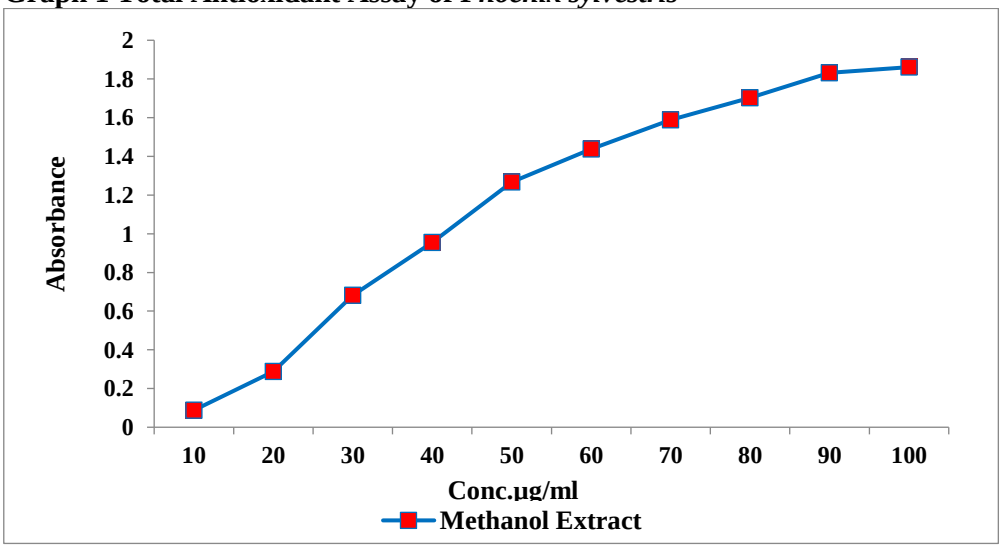
Table 3 Total Antioxidant Assay of *Phoenix sylvestris*

SI NO	Concentration (µg/ml)	Absorbance (695 nm)
1	10	0.088
2	20	0.288
3	30	0.681
4	40	0.954
5	50	1.268
6	60	1.438
7	70	1.589
8	80	1.703
9	90	1.832
10	100	1.861

Figure 2 Total Antioxidant Assay of *Phoenix sylvestris*



Graph 1 Total Antioxidant Assay of *Phoenix sylvestris*



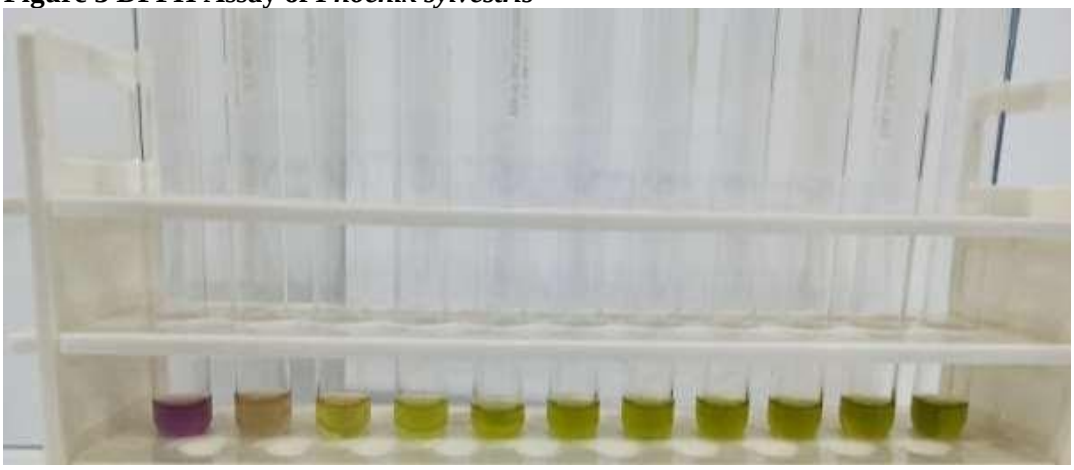
DPPH Assay

The methanol extract of *Phoenix sylvestris* demonstrated dose-dependent activity, reaching a maximum percentage of inhibition of 87.92% at a concentration of 200 µg/ml, in comparison to the standard quercetin, shown in the Table 4, Figure 3 & Graph 2.

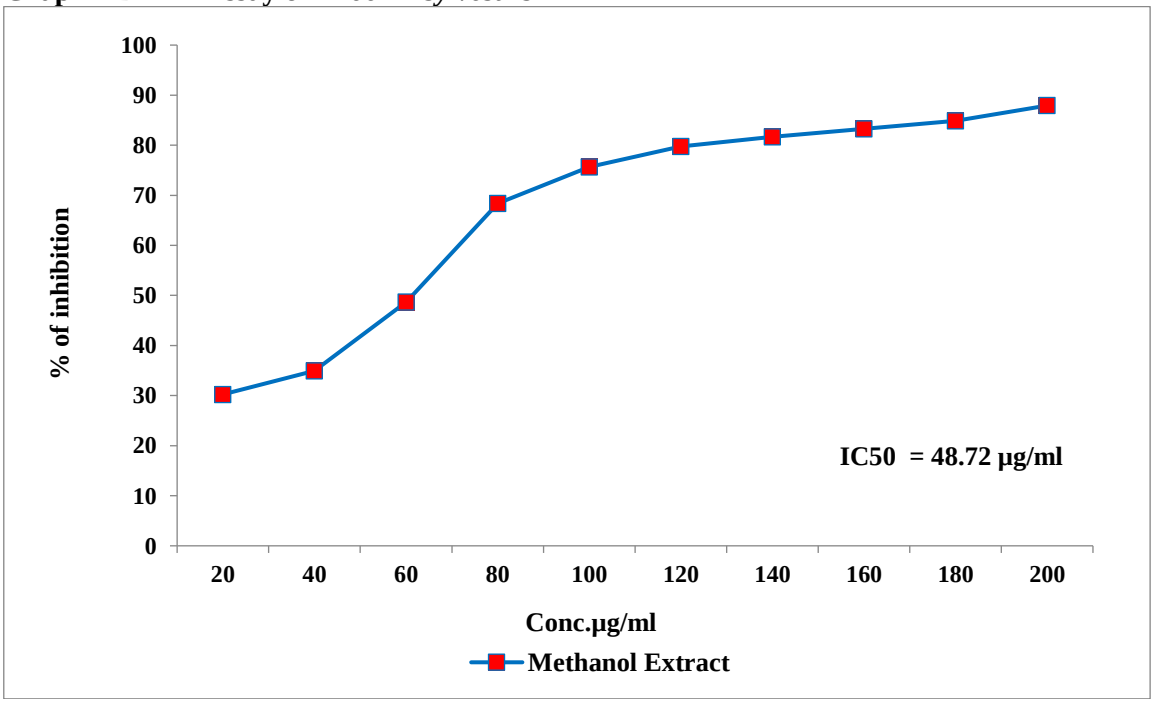
Table 4 DPPH Assay of *Phoenix sylvestris*

SI NO	Concentration (µg/ml)	DPPH Activity %
1	20	30.24
2	40	34.96
3	60	48.72
4	80	68.40
5	100	75.68
6	120	79.76
7	140	81.68
8	160	83.28
9	180	84.88
10	200	87.92

Figure 3 DPPH Assay of *Phoenix sylvestris*



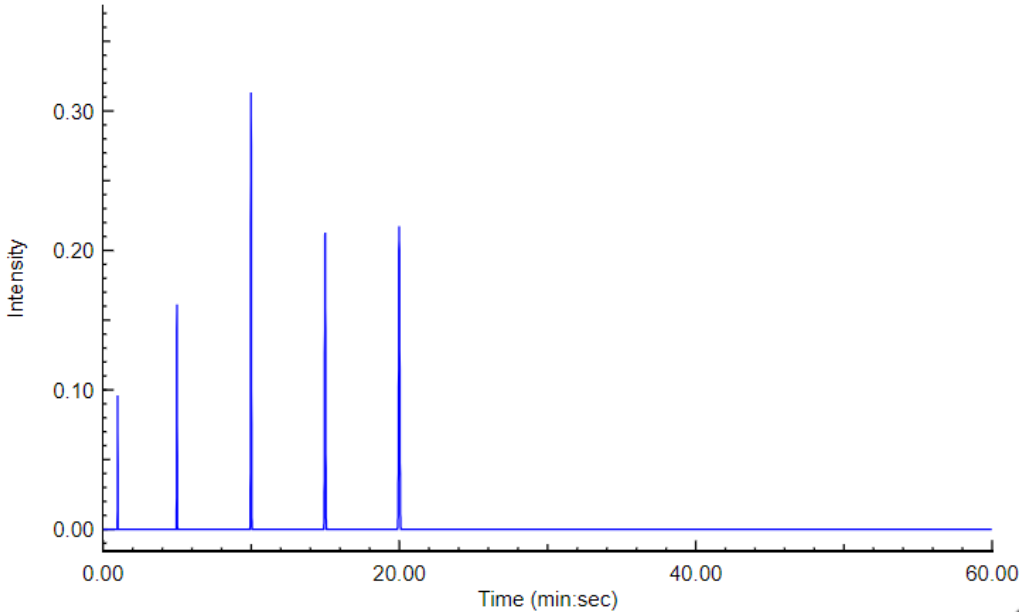
Graph 2 DPPH Assay of *Phoenix sylvestris*



Gas chromatography-mass spectrometry

The GC-MS analysis of the antioxidant compound obtained from the DPPH assay identified nerolidol as a significant constituent shown in the graph 3.

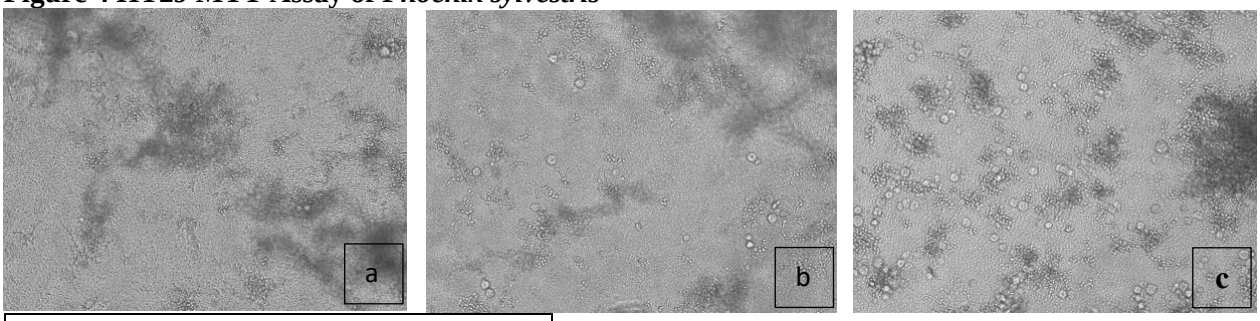
Graph 3 GC-MS



Anticancer Activity
Cell Lines
MTT Assay of HT29 Cell Lines

The methanol extract of *Phoenix sylvestris* exhibited varying degrees of inhibition against HT29 cells. The maximum percentage of inhibition, reaching 74.4%, was observed at a concentration of 50 µg/ml, while the lowest inhibition percentage, recorded at 10 µg/ml concentration, was 61.4%. These results are illustrated in the accompanying in the Table 5, Figure 4 & Graph 4.

Figure 4 HT29 MTT Assay of *Phoenix sylvestris*

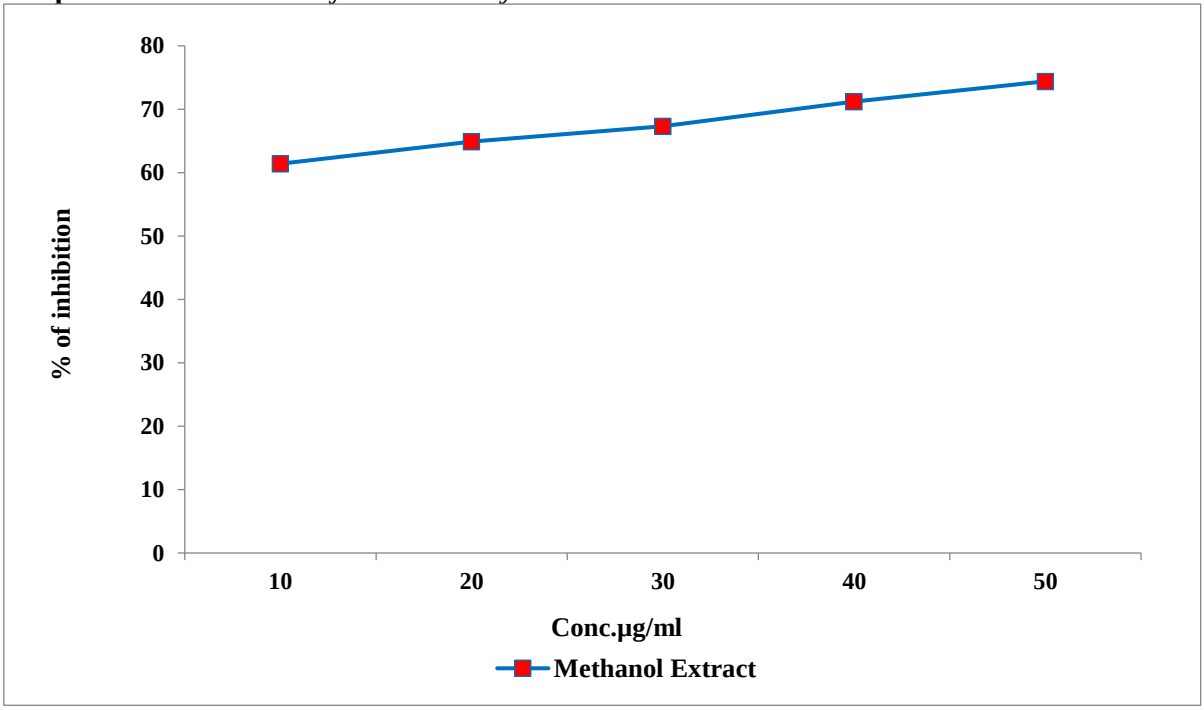


a. Control cells of HT29
b. Cells treated at 10µg/ml
c. Cells treated at 50µg/ml

Table 5 HT29 MTT Assay of *Phoenix sylvestris*

SI NO	Conc.µg/ml	% of inhibition
1	10	61.4
2	20	64.9
3	30	67.3
4	40	71.2
5	50	74.4

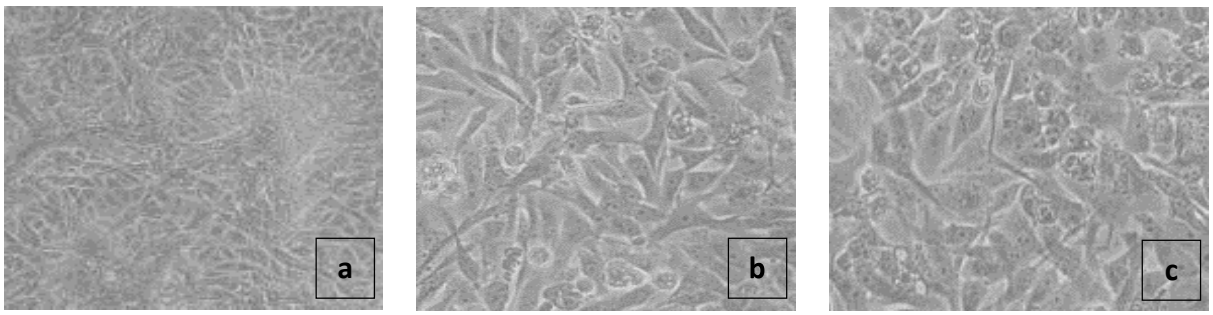
Graph 4 HT29 MTT Assay of *Phoenix sylvestris*



LDH Assay of HT29 Cell Lines

The methanol extract obtained from *Phoenix sylvestris* exhibited varying degrees of inhibition against HT29 cells. At a concentration of 50 µg/ml, the extract demonstrated the highest inhibition percentage, reaching 81.7%. Conversely, at a concentration of 10 µg/ml, the inhibition percentage was comparatively lower, measured at 52.8%, Shown in the Table 6, Figure 5 & Graph 5.

Figure 5 HT29 LDH Assay of *Phoenix sylvestris*

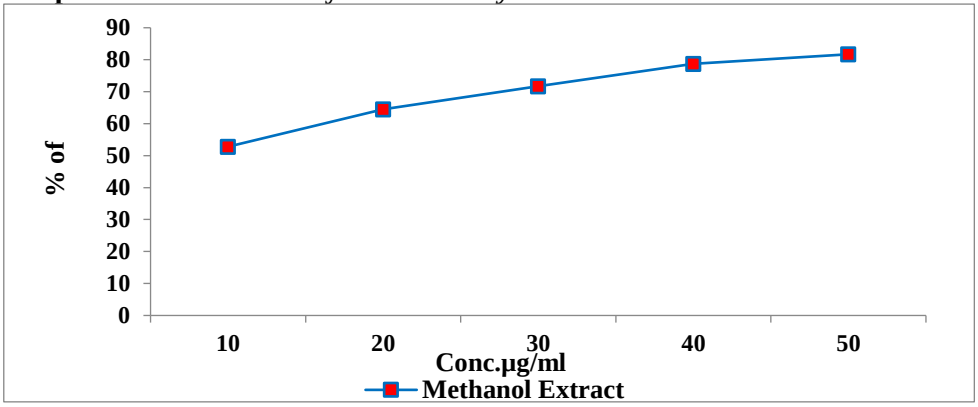


a. Control cells of HT29
b. Cells treated at 10µg/ml
c. Cells treated at 50µg/ml

Table 6 HT29 LDH Assay of *Phoenix sylvestris*

SI NO	Conc.µg/ml	% of inhibition
1	10	52.8
2	20	64.5
3	30	71.7
4	40	78.7
5	50	81.7

Graph 5 HT29 LDH Assay of *Phoenix sylvestris*



In silico analysis

Through molecular docking, Table 7 and Figure 6 present the binding interactions between GSK 3, AXIN, Porcine Osteocalcin, DVL & E-Cadherin with the Nerolidol, highlighting the optimal docking results.

Figure 6 Binding Interactions of Nerolidol

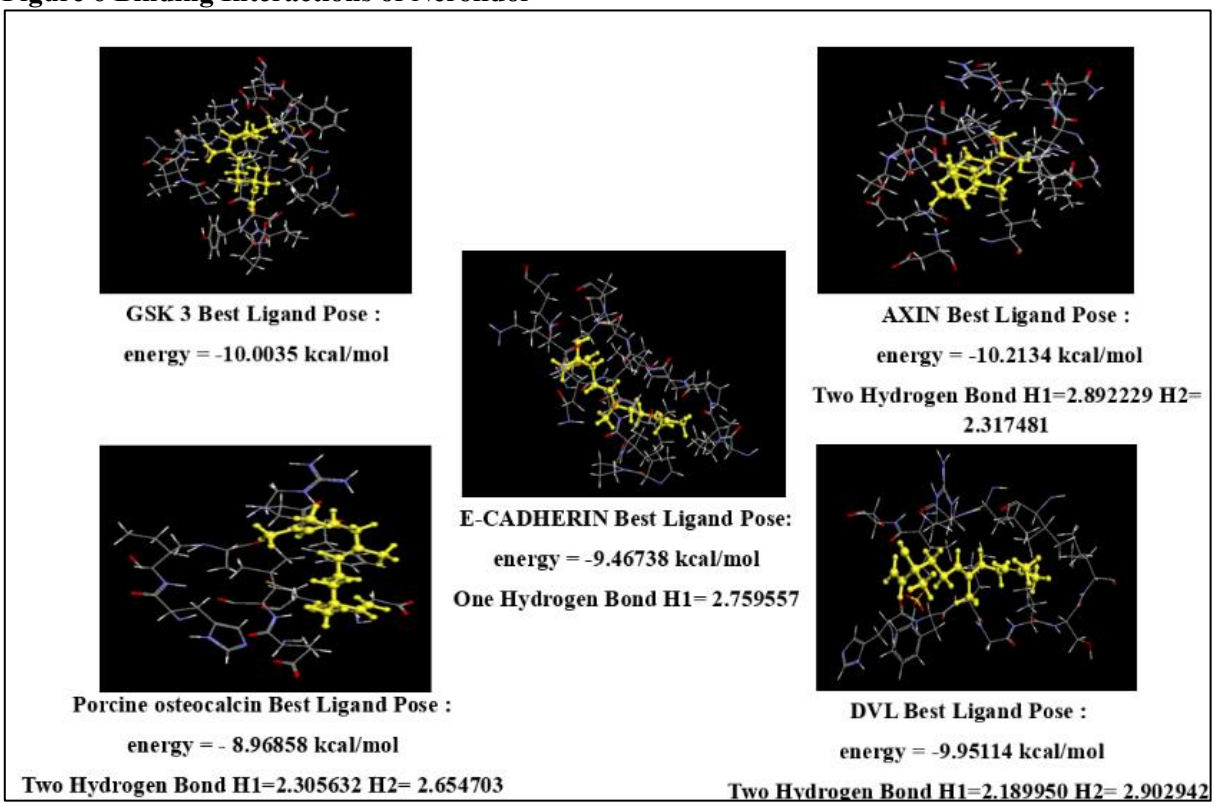


Table 7 Binding Interactions of Nerolidol

SI NO	Nerolidol interaction with Proteins	RCSB PDB IDs	NO. of Bonds	Bond length (A∘)	Binding energy (kcal/mol)
1	GSK 3	6HK3	-	-	**-10.0035**
2	Porcine Osteocalcin	1Q8H	2	2.305632	**-8.96858**
				2.654703	
3	E-Cadherin	4R11	1	2.759557	**-9.46738**
4	AXIN	2QZ5	2	2.892229	**-10.2134**
				2.317481	
5	DVL	3FY5	2	2.189950	**-9.95114**
				2.902942	

Discussion:

P. sylvestris has long been recognized for its medicinal qualities, historically employed in treating a variety of ailments including abdominal discomforts, fevers, unconsciousness, constipation, heart issues, toothaches, nervous weakness, and helminthiasis. Moreover, the plant exhibits a plethora of pharmacological activities, including antioxidant, anthelmintic, antimicrobial, cytotoxic, erythropoietic, antidiarrheal, analgesic, diuretic, anti-ulcer, antihypertensive, and antidiabetic properties. The plant is rich in vitamins, minerals, enzymes, amino acids, sugars (both reducing and non-reducing), tannins, proteins, steroids, and flavonoids. It serves as a valuable reservoir of natural antioxidants and dietary supplements, with its various plant parts extensively utilized as functional ingredients in the food sector. This study delves into the antioxidant and anticancer applications of Wild Date Palm (*Phoenix sylvestris Roxb*.).

Phytochemical analysis

Phytochemical analysis revealed the presence of various compounds known for their immunomodulatory, antimicrobial, anti-inflammatory, and anticancer properties (Kothari, 2011; Nag *et al*., 2012; Shukla *et al*., 2015). Sharma *et al*(2016) conducted phytochemical screening on crude hexane, dichloromethane, and methanol leaf extracts of *P. sylvestris*, revealing the presence of alkaloids, flavonoids, and phenols. This investigation also unveiled a diverse array of biologically active compounds including aldehydes, alcohols, flavonoids, phenolics, aromatic compounds, terpenoids, and fatty acid methyl esters.

GC-MS analysis

Sharma DC *et al* (2016), GC-MS analysis of the most powerful MPF8 revealed the presence of eight chemicals. Tetradecanoic acid, 2,6,10-Trimethyl,14-ethylene-14-pentadecene, Pentadecanoic acid, 2,4-Dimethoxybenzyl acetate, 2-hexadecen-1-ol, 3,7,11,15-Tetramethyl, 9-Octadecanoic acid, 2,4-Dimethoxybenzyl acetate, 2-hexadecen-1-ol, 3, depicts their % area and relative retention time. The synergistic effect of all the chemicals resulted in antioxidant, antibacterial, and other properties. In the GC-MS analysis, pentadecanoic acid had the highest percent area (52.90) and was previously found as having substantial anti-inflammatory, antibacterial, and anti-cancerous activity, whereas other substances were possessed strong protecting and curing properties, as discovered in a GC-MS analysis.

Antioxidant activity

The antioxidant activity of *P. sylvestris* leaves was evaluated in vitro. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable organic nitrogen radical. When DPPH reacts with phenol, it undergoes two distinct pathways: direct separation of a hydrogen atom from phenol and electron transfer. The outcome of these pathways is influenced by both the solvent used and the redox potential of the involved species. Antioxidants interact with DPPH, effectively neutralizing the free radical, resulting in a colour change in the reaction mixture from purple to yellow. The degree of discoloration indicates the potency of the antioxidant scavenging activity (Vladimir-Knežević *et al*., 2011).

Anticancer activity

Lamia FS *et al* (2021). The aqueous extract of Ajwa dates (*P. sylvestris*) was discovered to improve liver function by restoring antioxidant enzymes, liver enzymes, cytokines balance, and gene expression to normal levels. Because the treatment and prognosis of Hepatocellular carcinoma, one of the main causes of cancer-related deaths worldwide, is improved when the patient's liver function is preserved. As a result, in this review, we discussed the possibility of isolating potential anticancer agents from the extract of these fruits, which could then be used as an indigenous substance for the treatment of hepatocellular carcinoma. Khan F *et al* (2021), to test this hypothesis, we used a rat model of diethylnitrosamine (DEN) induced liver cancer to assess the HCC inhibitory effects and other positive features of the aqueous extract of ajwa dates (ADE). The reversion of DEN-damaged liver to normal was aided by ADE. The return to normal levels of antioxidant enzymes, liver enzymes, cytokines balance, and gene expression after ADE treatment suggests that ADE improves liver function and suppresses HCC. As a result, ADE can be administered in conjunction with other HCC treatments.

Isolation of Nerolidol

The *P. sylvestris* methanolic leaf extract of the plant was fractionated using a silica gel column to isolate the most promising fraction (MPF) and subsequently analyzed using Gas chromatography. the retention time as about 20minutes in gas column.

Docking analysis

Grid-based docking study was used to analyze the binding modes of molecules with the amino acids present in the active pocket of the protein. To identify the potential antidiabetic lead molecule, we have subjected the docking analysis of the active compounds of *Phoenix Sylvestris* (L.) to the active site of BACE1. In order to study the interaction of the compounds with alpha- amylase (PDB id: 1PPI). We performed Glide docking analysis by Schrodinger suite v10.1, where among of these compounds 4-methylcatechol shows highest docking score against both of the enzymes. Docking Score suggested that 4-methylcatechol had the highest affinity to the alpha-amylase corresponding to the other compound.

Conclusion:

The investigation into the methanol extract of Phoenix sylvestris has revealed a rich assortment of phytochemicals, including alkaloids, carbohydrates, saponins, proteins, amino acids, and phenolic compounds, though glycosides and terpenoids were notably absent. The extract displayed significant quantities of phenols, flavonoids, proteins, and carbohydrates, indicating its potential medicinal value. Additionally, the extract exhibited potent antioxidant activity and demonstrated dose-dependent inhibition against HT29 cells, suggesting its potential therapeutic application in combating oxidative stress and cancer. Further exploration of the extract's bioactive component of nerolidol and their interactions with specific molecular targets, such as GSK 3, AXIN, Porcine Osteocalcin, DVL, and E-Cadherin, could elucidate its mechanisms of action and enhance its pharmaceutical relevance.

References

1. Brody H. Colorectal cancer. Nature. 2015 May 14;521(7551):S1. doi: 10.1038/521S1a. PMID: 25970450.
2. Patel SG, Karlitz JJ, Yen T, Lieu CH, Boland CR. The rising tide of early-onset colorectal cancer: a comprehensive review of epidemiology, clinical features, biology, risk factors, prevention, and early detection. Lancet Gastroenterol Hepatol. 2022 Mar;7(3):262-274. doi: 10.1016/S2468-1253(21)00426-X. Epub 2022 Jan 26. PMID: 35090605.
3. Tonini V, Zanni M. Why is early detection of colon cancer still not possible in 2023? World J Gastroenterol. 2024 Jan 21;30(3):211-224. doi: 10.3748/wjg.v30.i3.211. PMID: 38314134; PMCID: PMC10835528.
4. Modest DP, Pant S, Sartore-Bianchi A. Treatment sequencing in metastatic colorectal cancer. Eur J Cancer. 2019 Mar;109:70-83. doi: 10.1016/j.ejca.2018.12.019. Epub 2019 Jan 25. PMID: 30690295.
5. Benson AB 3rd, Arnoletti JP, Bekaii-Saab T, Chan E, Chen YJ, Choti MA, Cooper HS, Dilawari RA, Engstrom PF, Enzinger PC, Fleshman JW Jr, Fuchs CS, Grem JL, Knol JA, Leong LA, Lin E, May KS, Mulcahy MF, Murphy K, Rohren E, Ryan DP, Saltz L, Sharma S, Shibata D, Skibber JM, Small W Jr, Sofocleous CT, Venook AP, Willett C; National Comprehensive Cancer Network. Colon cancer. J Natl Compr Canc Netw. 2011 Nov;9(11):1238-90. doi: 10.6004/jnccn.2011.0104. PMID: 22056656.
6. Chaluvadi SR, Young P, Thompson K, Bahri BA, Gajera B, Narayanan S, Krueger R, Bennetzen JL. *Phoenix* phylogeny, and analysis of genetic variation in a diverse collection of date palm (*Phoenix dactylifera*) and related species. Plant Divers. 2018 Dec 18;41(5):330-339. doi: 10.1016/j.pld.2018.11.005. PMID: 31934678; PMCID: PMC6951277.
7. W. M. Barnes. Proc. Natl. Acad. Sci. USA 91:2216, 1994. (2) N. A. Harrison *et al*. Plant Dis. 86:676, 2002. (3) N. A. Harrison *et al*. Ann. Appl. Biol. 153:85, 2008. (4) A. Jeyaprakash and M. A. Hoy. Insect Mol. Biol. 9:393, 2000.
8. Kuo P, Henderson IR, Lambing C. CTAB DNA Extraction and Genotyping-by-Sequencing to Map Meiotic Crossovers in Plants. Methods Mol Biol. 2022;2484:43-53. doi: 10.1007/978-1-0716-2253-7_4. PMID: 35461443.

9. Suganthi M, Abirami G, Jayanthi M, Kumar KA, Karuppanan K, Palanisamy S. A method for DNA extraction and molecular identification of Aphids. MethodsX. 2023 Feb 27;10:102100. doi: 10.1016/j.mex.2023.102100. PMID: 36915859; PMCID: PMC10006851.
10. Lee PY, Costumbrado J, Hsu CY, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. J Vis Exp. 2012 Apr 20;(62):3923. doi: 10.3791/3923. PMID: 22546956; PMCID: PMC4846332.
11. Sharma DC, Shukla R, Ali J, Sharma S, Bajpai P, Pathak N. Phytochemical evaluation, antioxidant assay, antibacterial activity and determination of cell viability (J774 and THP1 alpha cell lines) of P. sylvestris leaf crude and methanol purified fractions. EXCLI J. 2016 Feb 5;15:85-94. doi: 10.17179/excli2015-689. PMID: 27047320; PMCID: PMC4817419.
12. Sheikh ZA, Siddiqui ZA, Naveed S, Usmanghani K. Quantitative Determination of Catechin as Chemical Marker in Pediatric Polyherbal Syrup by HPLC/DAD. J Chromatogr Sci. 2016 Sep;54(8):1324-7. doi: 10.1093/chromsci/bmw078. Epub 2016 May 10. PMID: 27165575.
13. Azmat S, Zahoor A, Ifzal A, Ahmad VU, Mohammed FV. A new megastigmane glycoside, phoenixoside A, from Phoenix dactylifera. Nat Prod Commun. 2012;7:3–4.
14. Chowdhury, M. S. H. , Halim, M. , Muhammed, N. , Haque, F. , & Koike, M. (2008). Traditional utilization of wild date palm (*Phoenix sylvestris*) in rural Bangladesh: An approach to sustainable biodiversity management. Journal of Forestry Research, 19(3), 245–251.
15. Yan R, Cao Y, Yang B. HPLC-DPPH screening method for evaluation of antioxidant compounds extracted from Semen Oroxyli. Molecules. 2014 Apr 10;19(4):4409-17. doi: 10.3390/molecules19044409. Erratum in: Molecules. 2016;21(1). pii: E125. doi: 10.3390/molecules21010125. PMID: 24727414; PMCID: PMC6271040.
16. Flieger J, Flieger M. The [DPPH●/DPPH-H]-HPLC-DAD Method on Tracking the Antioxidant Activity of Pure Antioxidants and Goutweed (*Aegopodium podagraria* L.) Hydroalcoholic Extracts. Molecules. 2020 Dec 18;25(24):6005. doi: 10.3390/molecules25246005. PMID: 33353137; PMCID: PMC7766071.
17. Urquiza-López A, Álvarez-Rivera G, Ballesteros-Vivas D, Cifuentes A, Del Villar-Martínez AA. Metabolite Profiling of Rosemary Cell Lines with Antiproliferative Potential against Human HT-29 Colon Cancer Cells. Plant Foods Hum Nutr. 2021 Sep;76(3):319-325. doi: 10.1007/s11130-021-00892-w. Epub 2021 Jul 15. PMID: 34264453.
18. Jahani-Sherafat S, Azimirad M, Ghasemian-Safaei H, Ahmadi Amoli H, Moghim S, Sherkat G, Zali MR. The effect of intestinal microbiota metabolites on HT29 cell line using MTT method in patients with colorectal cancer. Gastroenterol Hepatol Bed Bench. 2019;12(Suppl1):S74-S79. PMID: 32099605; PMCID: PMC7011068.
19. Hu YL, Wang XB, Chen DD, Guo XJ, Yang QJ, Dong LH, Cheng L. Germanicol induces selective growth inhibitory effects in human colon HCT-116 and HT29 cancer cells through induction of apoptosis, cell cycle arrest and inhibition of cell migration. J BUON. 2016 May-Jun;21(3):626-32. PMID: 27569083.
20. Kaur T, Madgulkar A, Bhalekar M, Asgaonkar K. Molecular Docking in Formulation and Development. Curr Drug Discov Technol. 2019;16(1):30-39. doi: 10.2174/1570163815666180219112421. PMID: 29468973.
21. Pinzi L, Rastelli G. Molecular Docking: Shifting Paradigms in Drug Discovery. Int J Mol Sci. 2019 Sep 4;20(18):4331. doi: 10.3390/ijms20184331. PMID: 31487867; PMCID: PMC6769923.