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In vitro anticancer, antioxidant and DNA-binding study of the bioactive ingredient of clove and its isolation

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ABSTRACT

Cancer cannot be ignored since it is the most dangerous disease because it is a major cause of death globally with 15% mortality. Researchers have been attracted to the plant-based solution of this havoc. Among all plants, *Syzygium aromaticum* has shown tremendous results in many aspects such as anticancer, antioxidant, and others. All the studies that took place, were done on the plant extract only. No one goes further than this. Hence, an advanced computational chemistry-based method for the characterization and identification of the bioactive ingredients isolated from cloves was developed for the first time. First, different extracts of *Syzygium aromaticum* plant buds were obtained using different solvents (Water, methanol, chloroform, ethyl acetate, 50% ethanol, and hexane), then each extract was evaluated for its anticancer activity against A549 and H1299 lung cancer cell lines. The fractionation of the most active extract was done using flash chromatography. After that, anticancer evaluation of every fraction was done again. One of the obtained fractions showed the highest anticancer activity. For the identification of the most active fraction the experimental IR and NMR data of it was taken and compared with the computational IR and NMR data of 19 compounds found in cloves. Furthermore, DNA binding affinity and antioxidant activity of the fraction showing the highest anticancer activity were also studied. The presented method of the isolation of the most bioactive ingredient will be the most helpful for all the scientists working in the field of separation science and phytomedicine.

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1. Introduction

It cannot be denied that cancer is the most severe problem all over the world. Indeed, cancer is categorized by uncontrolled cell division leading to irregular evolution of the tissue [1]. Worldwide, cancer has become a cause of the deaths of 6.7 million people each year [2]. The most notable point is not only the detection of 18.1 million new cases of cancer but also 9.6 million deaths in 2018 due to cancer alone, as per Global Cancer Observatory (GLOBOCAN) approximation [3]. Therefore, there is a great need to fight against this fatal disease. Of course, there are many treatment methods, chemotherapy is considered the best because it works quickly. The main problem with chemotherapeutic agents is the attribution to cellular changes in cancer [4]. Besides, another problem that has put a question mark on the efficacy of chemotherapeutics, is the resistance of active cancer cells against chemotherapeutic agents. Moreover, chemotherapeutics not only show numerous serious side effects, but also have harmful effects on ordinary living cells of the body [5]. Therefore, it is essential to discover more actual natural medicines with fewer side effects to battle against cancer.

There are about 114,000 extracts of plants that have been tested by The National Cancer Institute for control of disease of cancer after collection of about 35,000 plant samples from 20 different countries. Besides, for cancer treatment, there are about 30-40% of the drugs obtained from plants [6-8] which are used worldwide. It is supposed that the usage of medicinal plants in the future, remains to hold significant potential for the prevention and cure of cancer [9]. Although the extracts of various herbs and medicinal plants have shown tremendous antitumor and antioxidant properties and have also been proved beyond doubt by laboratory experiments and clinical experiences [10], the use of plant extract raises many questions. For example, if we are using plant extract to cure any disease, it is very difficult to know which bioactive ingredient is actioning, and which one is not. Besides, the understanding of the mechanism of action also becomes very difficult while using the plant extract. It is because the plant extract has many phytochemicals, and which phytochemical is bioactive is unknown to everyone working with plant extract. Therefore, the currently developed method provides a systematic way to know the structure of the actual bioactive ingredients present in the extract. Among various plants, the flower buds of cloves (*Syzygium aromaticum* or *Caryophyllus aromaticus* L. or *Eugenia aromatica* or *Eugenia caryophyllus*) flower buds are found to be effective in the treatment of anodyne, carminative, anthelminthic, and cancer problems [11,12].

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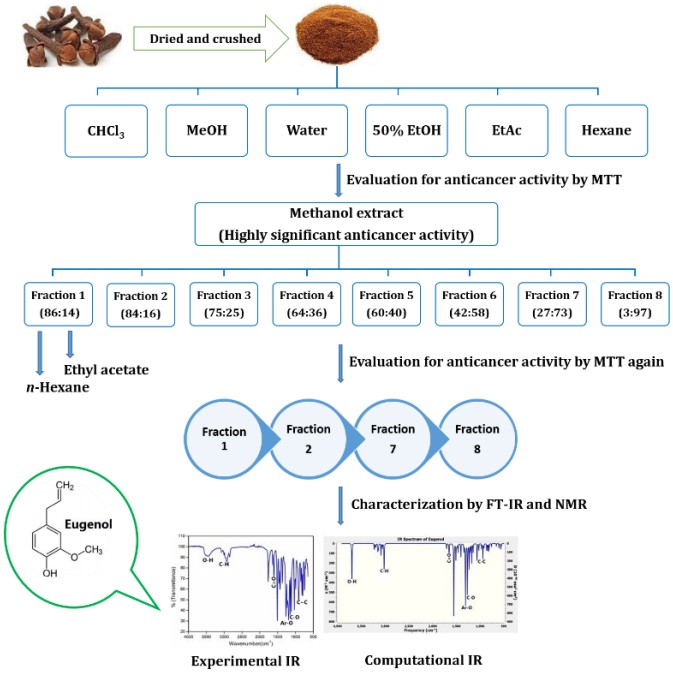


Figure 1. Extraction and isolation protocol.

During the literature survey, it was observed that there are some random studies on this plant to treat cancer disease, i.e., no systematic study was carried out. Besides, all studies that took place, were done on the plant extract only. No one goes further than this. Keeping these facts into consideration, I developed for the first time, a new and advanced method to display how to separate the most bioactive ingredients actioning against not only cancer but also other dangerous diseases. In the current study, the plant extracts were obtained using different solvents. After checking the anticancer activity of each extract against the cell lines, the extract showing the highest anticancer activity, was fractionated using Flash chromatography. After that, the anticancer activity of each fraction was checked again. Besides, the DNA binding and antioxidant studies of those fractions which showed better anticancer activity, were also carried out. Later, the FT-IR and NMR data of the fraction showing the highest anticancer activity was observed experimentally. To know the structure of the most active fraction, FT-IR and NMR data of all the phytochemicals found in clove as per GC-MS analysis report [13] was also generated computationally. In the last, both data (experimental and computational) were compared one by one to see what type of computational data was showing the similarity with the experimental data of the most active fraction. Thus, the structure of the most active ingredient was confirmed.

2. Experimental

2.1. Chemicals, software, and instruments

All solvents (Hexane ethanol, methanol, ethyl acetate, and chloroform), reagents, and chemicals used were of analytical grade, and were purchased from Merck, India. Millipore water was collected from the Millipore-Q, Bedford, MA, USA system. UV-vis spectrophotometer (Model T 80) PG Instruments Ltd., UK. Other types of equipment used were water bath, digital pH meter, sonicator, weighing balance, centrifuge machine, and biosafety cabinet class 2, CO_2 incubator, laminar air-flow, Elisa plate reader, microscope, Gaussian(R) 09 program [14] and GaussView 6.0 [15].

2.2. Preparation of clove bud powder and extraction

Syzygium aromaticum fresh flower buds were purchased from Khari Baoli Market, New Delhi, and validated through the Indian Agriculture Research Institute, Delhi, India, in the first week of September 2018. After drying, these buds were crushed to convert into fine small particles using a blender and stored in a desiccator at ambient temperature for extrac-tion purpose. After that, 50 g clove fine particles were saturated separately with 200 mL of different solvents (Water, methanol, 50% ethanol, ethyl acetate, chloroform, and hexane) in the Soxhlet apparatus (Figure 1). The temperature range during extraction was 70 to 110 °C, because different solvents have different boiling points. After putting for 72 hours, each extract was filtered with the help of Whatman No. 1 filter paper. The filtrate obtained was evaporated using a rotary vacuum evaporator. The solid residues were used to make the final solution before being transferred to a water bath for further drying. The stock solutions of 100 mg/mL of each extract were prepared for all six extracts and evaluated for anticancer activity studies separately.

2.3. Cell culture

The two non-small cell lung cancer cell lines (A549 and H1299), acquired from the National Center for Cell Sciences, Pune, were used to check the anticancer activity of each extract. Subsequently, these were sustained in DMEM medium, also known as a cell growth medium. This cell culture medium also comprises 10% FBS and 1% penicillin/streptomycin. The cells were cultivated and harvested in an incubator at 37±0.5 °C, 5% CO_2 and 95% humidity.

2.4. Treatment of cells

During the study, the cells were seeded in each well of 96-well plates in a medium containing 10% FBS and 1% penicillin/streptomycin to make it complete. After harvesting cells in an incubator for 24 hours, their medium was removed and the cells were substituted with fresh medium alone (control) or medium added with various concentrations of all six clove extracts. To

evade bias, the readings were taken two times. To ascertain the percentage of cell death, the yellow MTT assay was used [16].

2.5. Evaluation of the anticancer potential of Syzygium aromaticum extracts by MTT assay

The cells were grown in a CO_2 incubator for 24 hours with the media added with different concentrations of all six clove extracts of 50, 100, 200, 300, and 400 µg/mL. The solution of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used in concentration of 100 µg/mL (5 mg/mL). Cells were incubated for 24 hours, treated with 20 µL of MTT and again incubated for 4 hours at 37 °C. The mitochondria and dehydrogenases of the live cells were not damaged, which transformed the tetrazolium salt into insoluble crystals of formazan violet. Subsequently, 200 µL dimethyl sulfoxide (DMSO) was added to each well of the 96-well plate to dissolve these insoluble formazan crystals. In the last, the absorption microplate reader (*Microlisa plus*) was used to note the absorbance of each well at 570 nm (A570). The same method was used to verify the anticancer activity of all fractions obtained from methanol extract using flash chromatography.

2.6. Fractionation of methanol extract of Syzygium aromaticum

After witnessing the methanol extract as the most active extract, the fractions of the crude methanol extract were obtained on Flash chromatography with a binary and quarternary solvent delivery system, UV-Vis/Dual variable wavelength detector, Cheeta Purification Software (Bonna-Agela Technology, China.). For fractionations, 500 mg crude extract was mixed with silica to make its slurry, and loaded into the silica column (20 g, 40-60 µm). A mixture of ethyl acetate and *n*-hexane was used in the fractionation of the methanol extract. This mixture was allowed to flow through the column used in flash chromatography with a flow-rate of 30 mL/minute. First, the polarity of the mobile phase was maintained at zero, i.e., 100% *n*-hexane (nonpolar solvent) was allowed to flow through the column. After that, the fractions were obtained one by one (Figure 1) by increasing the amount of polar solvent (Ethyl acetate). The fractions obtained at different polarities are given in Table 1. Besides, each fraction of 30 mL was examined by TLC on silica gel using MeOH-$CHCl_3$ (1:9, v:v) as a mobile phase. The spots were detected and identified in a long UV cabinet.

Table 1. The definite polarity (ethyl acetate concentration) and the fractions obtained from the MeOH extract.

Fractions	Ethyl acetate percentage
Fraction 1st	14
Fraction 2nd	16
Fraction 3rd	25
Fraction 4th	36
Fraction 5th	40
Fraction 6th	58
Fraction 7th	73
Fraction 8th	97

2.7. Antioxidant potential of fractions of Syzygium aromaticum methanol extract

After gaining fractions of methanol extract, the antioxidant effect of each fraction was studied. The stable DPPH free radical, the antioxidant activities of the plant extract fractions were determined using the standard modified method [17]. Dilution of each fraction was done using methanol. Besides, 2.5 mL of MeOH was also used for ascorbic acid (2.5 mg) as standard. The solution of 0.004% DPPH, as a control, was prepared in MeOH. After that, 3.0 mL of the solution of DPPH was added to both 2.0 mL of sample and the standard of ascorbic acid solution, separately, and both were kept in the dark for 30 minutes. After that, with the help of UV-Vis spectrometer, the optical density of all solution mixtures was measured at 517 nm. The following formula was used to express the antioxidant activity in terms of % inhibition:

$$\text{DPPH scavenging effect (\%) or Percent inhibition} = (A_0 - A_1) \times 100 / A_0 \quad (1)$$

where A_0 and A_1 are denoting the absorbance of the control, and in the presence of test or standard sample, respectively. The blank solutions were used to correct values for radical decay. The solution of ascorbic acid was used as a reference standard solution. All the measurements were done in triplicate to avoid bias. After plotting the percentage inhibition values of the standard solution and sample solution, the IC_{50} of different solutions was also calculated.

2.8. DNA binding studies

The study of the interaction of four out of eight fractions of methanol extract with C_t-DNA was carried out at pH = 7.4 in a solution of *tris*-(hydroxymethyl)-aminomethane (Tris, 1×10^{-2} M) in double-distilled water. For the calculation of the concentration (30.0×10^{-4} M) of freshly prepared C_t-DNA at a wavelength of 260 nm (ε = 6600 $M^{-1}.cm^{-1}$), UV-Vis spectrophotometry was used at the starting of the experiment. After the concentration of DNA was confirmed, different solutions, having different concentrations of it, were made. First, the spectrum of the blank solution of Tris-HCl buffer at pH = 7.4, was taken at room temperature. To study the binding interactions, each fraction of methanol extract at a fixed concentration of 0.01 mg/mL, was mixed with different concentrations of DNA (30.0×10^{-4} to 1.0×10^{-5} M). To avoid bias, the titration experiments were repeated five times. A modified Benssi-Hilderbrand Equation [18] was applied for the calculation of the intrinsic binding constants (K_b). The following equation was used to determine the exact value of (K_b):

$$[DNA]/(\varepsilon_a-\varepsilon_f) = [DNA]/(\varepsilon_a-\varepsilon_f) + 1/K(\varepsilon_b-\varepsilon_f) -1 \quad (2)$$

where, absorption coefficients, ε_a, ε_f, and ε_b correspond to A_{obs}/[Plant fraction], extinction coefficient for the plant fraction in free form, and the extinction coefficient for the plant fraction in the fully bound form, respectively. The slopes and the intercepts of the plots of $[DNA]/(\varepsilon_a - \varepsilon_f)$ vs [DNA] were used for the calculation of the binding constants of different fractions. The protein-free nature of DNA, i.e., purity, was determined by considering the ratio of absorbance ratios (A260/A280 and A260/A230). It was found well within the range of 1.8-1.9 indicating the purity [18]. The additional DNA solutions were made from a stock solution (30×10^{-4} M) with a fixed concentrated solution of plant extracts (0.01 mg/mL).

2.9. Structure determination of the most active fraction

The structure determination of the fraction showing the highest anticancer and antioxidant properties was done with the help of FT-IR and NMR studies. Of course, the data obtained was unknown. Hence, the computational FT-IR and NMR data of 19 phytochemicals found in clove as per the literature data [13] was generated. IR data was generated using the DFT method in the ground state by using the Gaussian(R) 09 program, while NMR data was generated using ChemDraw software. For the generation of IR data, the basis set used was 6-311+G(d,p) using Becke's three-parameter hybrid functionals [20] with Lee, Yang, and Parr correlation functional method (B3LYP) [21]. The obtained IR data was visualized using GaussView 6.0. After that, the computational data (FT-IR and NMR) of 19 phytochemicals found in the clove was matched with experimental data (FT-IR and NMR) one by one.

Table 2. IC_{50} values of extracts of *Syzygium aromaticum* in different solvents.

Extracts	IC_{50} (cell line-A549), µg/mL	IC_{50} (cell line H-1299), µg/mL
Aqueous	3727.24	419.88
Methanol	292.34	113.41
Chloroform	3400.16	293.00
Ethyl acetate	3144.48	229.91
50% Ethanol	3059.36	106.50
Hexane	610.77	266.55

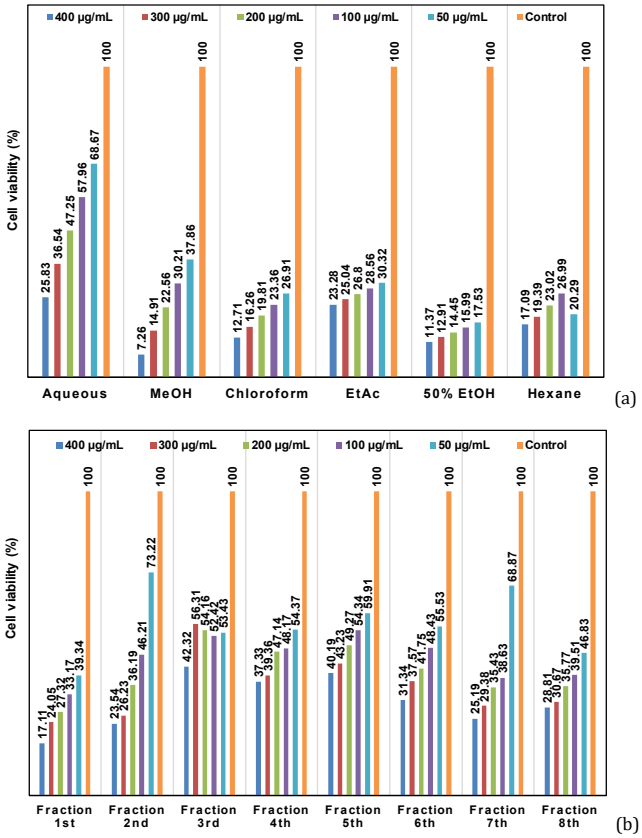


Figure 2. Anti-cancer activities of (a) clove extracts and (b) methanol extract fractions on A549 cell line.

Finally, the computational data of one of 19 phytochemicals, was found to have similarities with the experimental data of the most active fraction. Thus, the structure of the most active fraction was determined and confirmed.

3. Results and discussion

3.1. Extraction of the plant materials

During extraction using the Soxhlet apparatus, the colorless extracting solvents became colored, indicating the dissolution of the plant material constituents in the solvent. It was observed that the colors of the extracting solvents were different after the whole extraction. This observation indicated that the various solvents have different constituents of the plant materials. This was because of the varying polarities of the solvents used for extraction. After that, the obtained solutions were evaporated using a rotary evaporator apparatus to recover the extracting solvents in their colorless form.

3.2. Anticancer activity assay

The anticancer activities of six clove extracts were carried out on both H1299 and A549 cancer cell lines. The MTT assay was used to determine the anticancer activity of these different clove extracts. To evaluate and determine the cancer cell toxicity of these extracts, both cell lines were grown for 24 hours in complete media. After that, different concentrations, i.e., 50, 100, 200, 300, and 400 µg/mL of plant extract were added. On the other hand, cells in control wells were also grown in complete media only. A glance at Figures 2a and 3a reveals that the tested plant extracts displayed an anticancer effect on both cancer cell lines which is not dose-independent. The IC_{50} values were also calculated for all these extracts, which varied from 292 to 3727 µg/mL for A-549; 106 to 419 µg/mL for H-1299 (Table 2). These values confirmed that the methanol extract had more activity (IC_{50} = 113 and 292 µg/mL) in comparison to the other extracts. Therefore, methanol extract was selected for further studies by subjecting it to fractionations on flash chromatography.

3.3. Fractionation of methanol extract with flash chromatography

The fractions were obtained by flash chromatography, which was 8 in number (Table 1). These fractions were monitored by TLC. These fractions were further tested for their anticancer activities against A549 and H1299 cell lines. The results are shown in Figures 2b and 3b, respectively. It can be revealed from Figures 2b and 3b that among the eight fractions obtained by flash chromatography, only four fractions exhibited significant anticancer activity against A549 and H1299 cell lines.

Table 3. IC_{50} values of methanol fractions for A549 and H1299 cell lines.

Fractions	IC_{50} against A549 cell line, µg/mL	IC_{50} against H1299 cell line, µg/mL	Average IC_{50} against two cell lines (A549 & H1299), µg/mL
Fraction 1st	168.21	159.32	163.76
Fraction 2nd	142.52	184.16	163.34
Fraction 3rd	281.10	330.19	305.64
Fraction 4th	166.12	449.13	307.62
Fraction 5th	318.00	495.32	406.66
Fraction 6th	2879.80	128.31	1504.05
Fraction 7th	213.11	168.79	190.95
Fraction 8th	145.90	231.01	188.45

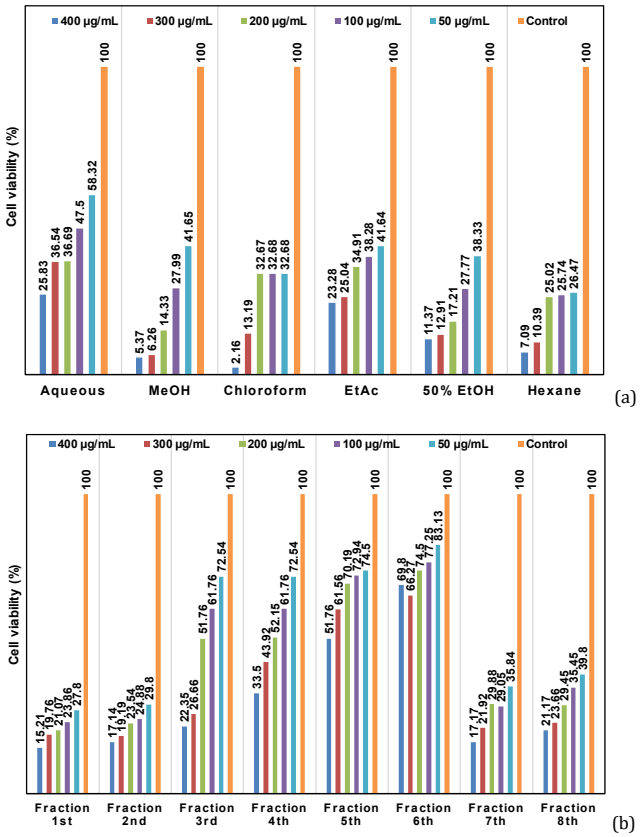


Figure 3. Anti-cancer activities of (a) clove extracts and (b) methanol extract fractions on H-1299 cell line.

The IC_{50} values for all these fractions are calculated (Table 3). The IC_{50} values of all these fractions ranged from 142.5 to 2879.8 µg/mL. Furthermore, only four fractions out of eight exhibited significant dose-related anticancer activity against two cell lines, A549 and H1299. These fractions were known as active fractions 1st, 2nd, 7th, and 8th, respectively. In these fractions, the 1st colorless fraction gave tremendous results, i.e. showed the highest not only anticancer activity but also antioxidant activity.

3.4. Antioxidant activity assay

During this study, the radical scavenging effects of active fractions, i.e., 1st, 2nd, 7th, and 8th, were calculated. All tested fractions exhibited scavenging effects on DPPH (Table 4). It was noted that the scavenging activities of the fractions of *Syzygium aromaticum* buds at different concentrations (1.56 to 400 µg/mL), were quite good (26-97%). The inhibitory activities observed were 97.0, 53.0, 56.0 and 57.0% for fractions 1st, 2nd, 7th and 8th, respectively. The highest antioxidant property was observed with respect to the antioxidant activities of ascorbic acid (28-90%). It was observed that the fraction 1st had greater antioxidant activity than not only ascorbic acid but also the other three fractions. Of course, different antioxidant activities were due to various constituents. These results in terms of IC_{50} values showed the scavenging activities of the selected fractions of methanol extract on DPPH radicals at various concentrations. The IC_{50} values calculated for fractions 1st, 2nd, 7th, and 8th were 2.80, 126.50, 251.0, and 251.0 µg/mL.

3.5. DNA binding study

DNA binding studies of methanol extract fractions were carried out as described above. Electronic absorption bands of fractions of the most active plant extract (MeOH extract) in the absence and presence of DNA are recorded and shown in Figures 4a-d. The different calculated parameters of interactions of plant fractions with DNA are depicted in Table 5. The parameters include λ_{max} (nm), free plant extracts (λ_f), plant extract fractions bonded to DNA (λ_b), change in wavelengths after binding with DNA ($\Delta\lambda_{max}$), the absorbance of free plant extract fractions (A_f), absorbance of plant extract fractions bonded to DNA (A_b), and change in absorbance after binding with DNA (ΔA).

The comparison of λ_{max} values of methanol extract fractions, and after binding with DNA (230 to 290 nm) confirmed hypochromism Figures 4 and 5. These figures also confirm bathochromism (blue shift) in the case of all fractions; hypochromism was observed in all fractions, except one, i.e. 7th that showed hyperchromism.

Table 4. Percentage inhibition of different fractions of *Syzygium aromaticum* with ascorbic acid as control.

Concentrations (µg/mL)	Ascorbic acid (% Inhibition)	Fraction 1st (% Inhibition)	Fraction 2nd (% Inhibition)	Fraction 7th (% Inhibition)	Fraction 8th (% Inhibition)
1.56	28	26	40	28	31
3.12	36	31	60	31	36
6.25	44	32	63	38	40
12.50	68	33	76	40	40
25	80	34	87	41	43
50	90	45	93	42	43
100	90	97	96	47	48
200	90	97	96	53	49
400	90	97	97	56	57

Table 5. DNA binding study of fractions of MeOH plant extract *.

Plant Extracts	λ_f (nm)	λ_b (nm)	$\Delta\lambda_{max}$ (nm)	A_f	A_b	ΔA	H%	K_b (M^{-1})
Fraction 1st	254	250	4.0	0.10	0.15	0.05	50	25×10^5
Fraction 2nd	256	256	0.0	0.07	0.11	0.04	36	24×10^5
Fraction 7th	258	259	1.0	1.50	0.30	0.12	80	75×10^4
Fraction 8th	258	258	0.0	2.10	0.20	1.90	90	28×10^4

* % Hypo/Hyperchromism (H%) = [Change in absorbance /A_f]×100, where A_f and A_b represent the absorbance of free and bound extract fractions; $\lambda_f = \lambda_{max}$ (free fractions), $\lambda_b = \lambda_{max}$ (fractions bound to DNA) and K_b = Binding constant.

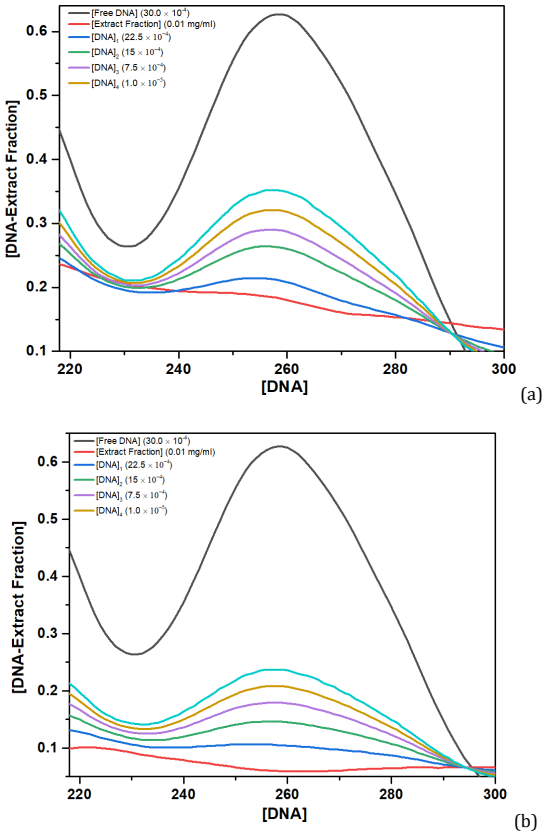


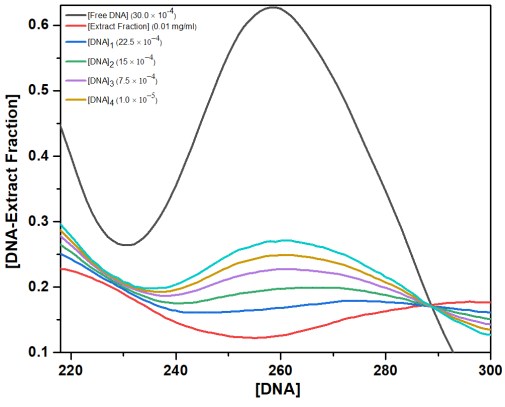
Figure 4. DNA binding spectra of (a) fraction 1st and (b) fraction 2n.

The observed data were also used to determine the intrinsic binding constant (Kb) [17]. The marked changes were observed in the absorption bands by the addition of increasing concentrations of DNA to the plant fractions. These observations can be explained by the process of π-electrons combination of fractions with π electrons of DNA bases. According to Table 5, the order of the binding constant of these fractions was 1st > 2nd > 8th > 7th.

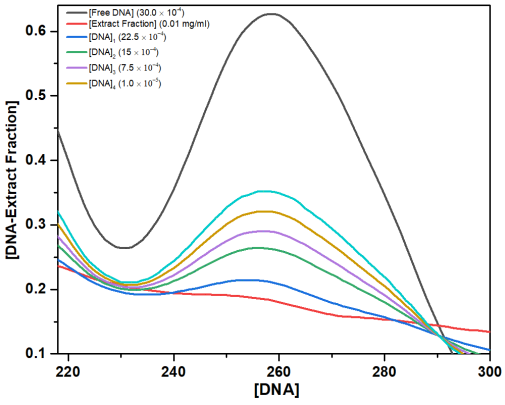
At the same time, fraction 2nd may be having charges moieties. These positively charged moieties are predicted to flexibly and directly affect DNA binding in the form of classical electrostatic interactions. In addition, there are negatively charged oxygens in the phosphate group of DNA, which may also have electrostatic interactions and eventually lead to further spectral changes. These results clearly show the attachment of the fraction to DNA in outside grooves [22,23]. In addition, more interactions such as hydrogen bonds between plant fractions and base pairs in DNA may also be present. All these interactions discussed can not only counter the major hypochromic effect but also have a deteriorating impact on it.

3.6. Structure confirmation of the most active fraction

When the computational IR and NMR data of 19 phytochemicals was matched with the experimental IR and NMR data of fraction 1st, the compound whose data was matching with that of fraction 1st, was eugenol. Additionally, the mass spectrometry of the fraction displayed a molecular ion peak $[M-H]^+$ with *m/z* 163.05 as the calculated mass of the fraction is 164.2.



(a)



(b)

Figure 5. DNA binding spectra of (a) fraction 7th and (b) fraction 8th.

The experimental and computational data, using of which the structure of eugenol was confirmed (Experimental data: Boiling point: 254-256 °C. Color: Pale-yellow oil. FT-IR (ν, cm^{-1}): 3442 (free OH strec.), 1636 (allylic group, strec.), 1190 (C-O-C strong strec.), 2925 (Ar C-H strec.), 1510 (C=C aromatic strec.). ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm): 3.395 (d, 2H, >CH_2), 3.835 (s, 3H, OMe), 5.550 (s, 1H, OH), 5.973 (multiplet, 1H, olefinic), 5.077 (dd, 1H, olefinic {germinal proton}), 5.110 (dd, 1H, olefinic {geminal proton}) 6.706 (d, 1H, Aromatic), 6.966 (dd, 1H, Aromatic). ESI-MS (*m/z*) calcd. for $C_{10}H_{12}O_2$ [M-H]$^+$: 164.2, found: 163.05. Computational data: Peak information: 3778 (free OH strec.), 1648 (allylic group, strec.), 1204 (C-O-C strong strec.), 3019 (Ar C-H strec.), 1509.77 (C=C aromatic strec.).)

4. Conclusions

Based on results and discussion, it can be concluded that the currently developed method is new, helpful, and systematic which involves not only experimental data but also computational data for the confirmation of the structure of the isolated bioactive phytochemical. Of course, the isolation of bioactive ingredients present in the plant extract is a challenging task, which the presented method resolves. If the extract of any plant is showing biological activity, but it is difficult to know about the most active ingredient, the current method will help definitely in the observation and confirmation of it. Moreover, the presented method may also be followed by other scientists working in the field of separation science. It is because any well-known bioactive phytochemical from any plant can be separated by following the procedure pronounced in the current study. Therefore, the currently developed method will be very useful to the scientific community.

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Disclosure statement

Conflict of interest: The author declares that he has no conflict of interest.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the author.
Human and animal rights: No animals/humans were used for the studies that are the basis of this research.

CRediT authorship contribution statement

Conceptualization: Mohammad Suhail; Methodology: Mohammad Suhail; Software: Mohammad Suhail; Validation: Mohammad Suhail; Formal Analysis: Mohammad Suhail; Investigation: Mohammad Suhail; Resources: Mohammad Suhail; Data Curation: Mohammad Suhail; Writing - Original Draft: Mohammad Suhail; Writing - Review and Editing: Mohammad Suhail; Visualization: Mohammad Suhail; Funding acquisition: Mohammad Suhail; Supervision: Mohammad Suhail; Project Administration: Mohammad Suhail.

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