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Phytochemical screening, Antioxidant and Anticancer activities of methanolic Extracts of *Syzygium aromaticum* (L.) Merr. and L.M.Perry

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ABSTRACT
In this study, to understand the antioxidant and anticancer activities of Syzygium aromaticum, the flower bud of S. aromaticum was subjected to methanol extraction by using maceration extraction technique, then it was subjected into antioxident activities and anticancer activity against human lung cancer cell lines by using MTT assy. S. aromaticum showed quantified amount of primary phytochemicals such as total phenol, tannin, saponin, and flavonoids, GC-MS results revealed, totally 14 secondary bioactive components, 5-phenyl-thiazolidine, 3,6-dimethyl-2-nitrobenzaldehyde, 3-hexyn-2-ol, 5-methyl, Cyclopenta [c]furo [3', 2':4,5] furo [2,3-h] n-decanoic acid, [1] benzopyran-11 (1 h)-one 3,4-anhydro-d-galactosan, 3-decyn-2-ol, 2,3-epoxyhexanol, bicyclo [2.2.1] heptan-2-one, 4,7,7-trimethyl-, semicarbazone, hexatriacontane, 1-iodo-2-methylundecane, spiro [androst-5-ene-17,1'-cyclobutan] -2'-one, 3-hydroxy, 2-amininononadecane, 2-methyl-6-methylene-octa-1,7-dien-3-ol were detected. The flower bud extract showed good amount of antioxidant activities such as DPPH, FRAP, total antioxidant and metal chelating activities, along with it also showed a significant level of cytotoxicity activity against A549 lung cancer cell line when compared with control. Based on the above results, S. aromaticum flower buds showed good antioxident and anticancer activities, so it could be considered as the most valuable alternative medicinal source for many diseases.
Keywords: *Syzygium aromaticum*; Flower buds; GC-MS; Antioxidant; Cytotoxicity *lung cancer.*

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INTRODUCTION

Traditional medicinal systems based on the use of herbal remedies, such as Ayurveda, Chinese, and Egyptian, continue to play an important role in health care systems. It is estimated that 40% of the world population depends directly on plant-based medicine for their health care. India has a rich medicinal flora of some 25,000 species, out of which 150 species are commercially used for extracting medicines or drug formulations [1]. In recent decades, medicinal plants have been gaining wider acceptance due to the perception that these plants, being natural products, have fewer side effects and improved efficacy than their synthetic counterparts [2,3]. *Syzygium aromaticum* L. also known as clove, is a dried flower bud belonging to the Myrtaceae family that is indigenous to the Maluku islands in Indonesia but has recently been farmed in different places worldwide [4, 5]. Interestingly, they are commercially used for many medicinal purposes and in the perfume industry, cloves are considered one of the spices that can be potentially used as preservatives in many foods, especially in meat processing, to replace chemical preservatives due to their antioxidant and antimicrobial properties [4,6]. Several reports have documented the antibacterial, antiviral, anticarcinogenic, and antifungal activities of some aromatic herbs, including cinnamon, oregano, clove, thyme, and mint. However, clove has gained much attention among other spices due to its potent antimicrobial and antioxidant activities [7]. Antioxidants are known to scavenge free radicals and singlet oxygen that can make all cellular components appear to be sensitive to oxidative damage [8]. Every branch of medicine and biological sciences recognised antioxidants as beneficial to

health and disease of the free radicals come from oxygen atoms called reactive oxygen species (ROS). It belongs to the superoxide ion (O_2), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2) classes [9]. On the other hand, free radicals are important biochemical responses in the body that are involved in numerous diseases such as inflammation, diabetes, cancer, heart disease, aging, neurodegenerative, and immunosuppression disorders [10]. The natural phytochemicals based on antioxidants like phenolics, flavonoids, alkaloids, vitamins and minerals play a key function in defending cells and tissues from oxidative damage. These natural antioxidants have been found in fruits, veget ables, and spices, and have the potential for free radical scavengers that are associated with a lower risk of inflammation and cancer [11]. The International Agency for Research on Cancer estimates that there are approximately 17 million cancer deaths each year and that there will be approximately 26 million new cancer cases by 2030 [12,13]. There has been a broad need for cancer related research in plant-based products. The aim of the present study is to examine the antioxidant and anticancer properties of flower bud extract of *S. aromaticum*.

MATERIAL AND METHODS

Collection of plant material:

The *Syzygium aromaticum* was purchased from local market from Salem, TamilNadu, and it ground into a fine powder then it stored at 4°C in sterilized plastic containers for further usage.

Extract preparation:

The dried flower bud sample (*S. aromaticum*) was subjected into methanol extraction by using the maceration extraction technique, using an orbital shaker at 90 rpm for 24 h. The solvents were evaporated by a rotary vacuum evaporator at 45°C. The dark, gummy like solid extracts were obtained for further studies.

Determination of Total Phenol:

The quantification of total phenols was determined as described method [14]. The 200 µL of methanol extracts were taken into test tubes and 500 µL of folin-ciocalteau phenol reagent (1N) was added to all the test tubes including the blank. After 5 min, 2.5 ml of sodium carbonate (20%) was added to all the test tubes. The test tubes were mixed well and incubated in dark for 40 min. The formation of blue color in the incubated test tubes indicated the presence of phenolics. Soon after incubation, the absorbance was read at 725 nm against the reagent blank. The results were expressed as Gallic acid equivalents (GAE). The analyses were performed in triplicates.

Determination of Tannin:

The tannin content of the sample was determined according to this method [14]. The 200 µL of each extract was incubated with 100 mg of polyvinyl polypyrrolidone (PVPP) and 500µL of distilled water for 4h at 4^0C. After incubation, the Eppendorf tubes were centrifuged at 3000 rpm for 10 min at 4^0C. The supernatant contains only the non-tannin phenolics since the tannins would have been precipitated along with PVPP. The supernatant was collected and phenolics were determined by the same method described for the quantification. The 100 µL of extracts were taken in a test tube and 500 µL of folin-ciocalteau phenol reagent (1N) were added to all the test tubes including the blank. After 5 min, 2.5 mL of sodium carbonate (20%) was added to all the test tubes. The test tubes were vortexed well to blend the contents and incubated in dark for 40 min. The formation of blue color in the incubated test tubes indicated the presence of phenolics. Soon after incubation, the absorbance was read at 725 nm against the reagent blank. The analyses were also performed in triplicates and the results were expressed in Gallic acid equivalents. From these two results, the tannin content of the plant samples was calculated as follows,

Tannins= total phenolics - non phenolics

Determination of Total Saponin:

The total saponin content of the sample was determined by the method [15]. The 0.2 mL of extract was taken to which 0.25 mL vanillin reagent (8% vanillin in ethanol) and 2.5 mL of 72% aqueous H_2SO_4 were added. The reaction mixtures in the tubes were heated in a water bath at 60°C for 10 min. After those tubes were cooled in ice for 4 min and then allowed to cool at room temperature, the absorbance was read at 544 nm. Diosgenin was used as a standard and the results obtained were expressed as mg diosgenin equivalent per g of sample dry matter.

Determination of Total Flavonoids:

The total flavonoid was determined by aluminium trichloride methode [16]. A volume of 200 µL of the extract was taken in test tubes and 2 mL of distilled water was added to each test tube. A tube contained 2.5 mL of distilled water served as a blank. To this 150 µL, 5% of $NaNO_2$ was added to all the test tubes and incubated the whole mixture for 6 mins at room temperature. After incubation, 150 µL of 10% $AlCl_3$ was added to all the test tubes including the blank. Then, the test tubes were incubated for 6 min at room temperature. Then 2 mL of 4% NaOH was added and made upto 5 mL using distilled water. The contents

in all the test tubes were mixed well and allowed to stand for 15 min at room temperature. The development of pink color due to the presence of flavonoids was read spectrophotometrically against prepared reagent blank at 510 nm. Samples were analyzed in triplicates and the amounts of flavonoids were expressed in Rutin equivalents.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis:

The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1µl of *S. aromaticum* extract sample injected into the instrument the oven temperature was as follows: 60°C (2 min); followed by 300° at the rate of 10C° min^{-1}; and 300°C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 240°; ion source temperature 240°C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

Antioxidant activities:

Determination of α, α-diphenyl-β-picrylhydrazyl (DPPH) assay:

The free radical scavenging activity of the extracts was done using the 1,1-diphenyl-2-picrylhydrazyl test according to this method [17]. The 0.2 ml extract was added to 3 ml of 0.004% methanol solution of DPPH. Absorbance was taken at 517 nm after 30 min and the percentage inhibition activity was calculated from $[(A_0–A_1)/A_0]$ x100, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard.

Determination of total antioxidant assay:

The antioxidant power of the extracts has been assayed with the phosphomolybdenum reduction method [18]. It was based on the reduction of the extract and subsequent formation of a complex (green color) at acidic pH, 200 µl of an extract with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 min at 695 nm using spectrophotometer against blank.

Determination of Ferric Reducing Antioxidant Power (FRAP) assay:

The FRAP assay was done according to this method [19], with some modifications, stock solutions included 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3$ $6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 ml $FeCl_3$ $6H_2O$ solution and then warmed at 37° before use. 200 µL of extracts were allowed to react with 2850 µL of the FRAP solution for 30 min in the dark condition. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The result was expressed in mM TE/g fresh mass.

Metal chelating activity assay:

The metal chelating assay was performed based on this method [20]. The metal chelating activity of extract, ferrous ions Fe^{2+} was measured according to the method previously described. To various concentrations 20, 40, 60, 80 and 100µL of extract in 1.6 mL of deionized water and 0.05 mL of $FeCl_2$ (2 mM) were added. After 30 sec, 0.1 mL ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}-Ferrozine complex was measured at 562 nm. EDTA was used as the standard metal chelating agent and the results were expressed as mg EDTA equivalents/g extract.

Cell culture condition:

The Human lung cancer cell A549 was procured from the National Center for Cell Sciences (NCCS), Pune, India. The cells were maintained in McCoy's 5A medium supplemented with FCS and 2 mM l-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na2CO3, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 1.5 g/L glucose, 10 mM (4-(2- hydroxyethyl)-1- piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin (100 IU/100 µg) were adjusted to 1 mL/L. The cells were maintained at 37^0C with 5% CO_2 atmosphere.

MTT assay:

A549 cell lines were seeded in 96-well microtiter plates (2000 cells/well) in DMEM medium [supplemented with 10% FBS (v/v), and 1% L-glutamine 200 mM (w/v)]. After one day of culture, the medium was removed by aspiration and the cells were treated with different concentrations (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/ml) of methanol extracted compounds in 100 µl DMEM medium. Following 24 h of incubation at 37°C, to each well added 100 µL (2 mg/ml) of 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (Sigma, USA) and incubated for another 3 h at 37°C. Finally, to all the wells added 100 µl of DMSO to lyse the cells and the absorbance of the cell lysates was

measured at 540 nm using a microplate reader (Dynatech MR 5000, Dynatech laboratories, USA). Similarly, the control wells contained DMSO in culture medium and the blank contained only DMEM [21].

Statistical analysis:

The results were analyzed by one way ANOVA to calculate the significance of difference among the treatments. This data were analyzed statistically using by IBM SPSS 20 software.

RESULTS AND DISCUSSION

Primary Phytochemical Analysis of *S. aromaticum* :

The primary phytochemicals such as total phenol, tannin, total saponin and total flavonoids were found to be flower bud methanol extraction of *S. aromaticum*, among them, more amount of total phenols was observed in methanolic extracts of *S. aromaticum* when compared with others (Fig.1). *E. caryophyllata* showed various phytochemical such as terpenoids, tannins, saponins, flavonoids, tannins, saponins, and flavonoids were observed in different solvent extracts [22]. The *S. aromaticum* has huge amount of phytochemicals as by Sesquiterpenes, monoterpenes Phenolic compounds and eugenol followed mostly significant in clove oil [23]. The secondary metabolites include phenol, flavonoid, tannins, alkaloids, sterols, glycosides, to relationship between great therapeutic values were largely used for drugs and pharmaceutical products [24,25].

Secondary Phytochemical Analysis of *S. aromaticum*:

The GC-MS analysis in *S. aromaticum* flower bud methanol extraction was analyzed, based on the estimation of secondary phytochemicals. It revealed that presence of various Secondary phytocompounds were could contribute the medicinal values, the identification of the phytochemical compounds were confirmed based on the peak area (%), Retention time (RT) and molecular formula (MW). The highest peak area % was found to be in 3-decyn-2-ol through GC-MS analysis when compared with other compounds (Table 1 and Fig 2). The GC-MS investigation of clove oil is main compounds in eugenol, β-caryophyllene, eugenol acetate [26]. The chemical profile analysis was evaluated by GC-MS the determined five major compounds related for eugenol like compounds form *S. aromaticum* essential oil [27]. Pharmacology documentation of clove flower buds were major sources of Phenolic compound and flavonoids 18% of the presence of essential oil [23].

Antioxidant activities:

In the present study, good amount of antioxident activities, total antioxidant and DPPH radical were observed, followed by FRAP and metal chelating activities Fig-3. The free radical scavenging action may be due to the presence of phenolic, tannin, flavonoids and saponin components in *S. aromaticum* flower bud methanolic extracts. In the presence study *S. aromaticum* flower bud methanol extracts has been showed high level of phenolics content that could be naturally free radical terminators. The *E. caryophyllus* various solvent extract were performed by DPPH radical scavenging activity observed at different concentration 20-200 µg/ml range. The methanolic extracts showed better activity that compared to α-tocopherol standard [28]. The *S. aromaticum* flower (cloves) good level of Phenolic compounds do to the relevant for antioxidant properties. The early reported to The main assets of phenolic compounds were located in cloves as Phenolic volatile oils like eugenol, acetyl eugenols and tannins, for great ability of radical scavengers [4]. The antioxidant activity of clove treated rat model showed that protected role against H_2O_2 might be due to the effect of essential oil active compounds [29, 1]. The flavonoids, tannins and alkaloids has a variety of medicinal purpose especially antioxidant and anti-inflammatory activities. The biological functions of saponins were anti-fungi, insecticidal, phytotoxic and hemolytic activity [30,31]. The presence of bioactive compounds by *caesalpinia sappan* like phenol and flavonoid responded antioxidant and anticancer activities [32]. The plants defend themselves by producing some compounds of various chemical natures called as secondary metabolites [33]. Phenoic compounds contain high level of antioxidant properties [34].

Anticancer activity:

The examined effects on the A549 lung cancer cell line by using MTT assay. Fig 4 showed the percentage of cell viability of crude methanolic extract was treated with various concentrations such as 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 mg/ml respectively, against human lung cancer cell line. After 24h of treatment, cell viability become decreased when increase the concentrations, the microscopic observation revealed that morphological changes, cell shrinkage, destruction in chromatin clumping were observed when compared with untreated cells Fig 5. The investigational results was established that this plant extract has ability to inhibit the cell proliferation in a dosage dependent against A549 lung cancer cells. The *Pandanus canaranus* leaf extract was against human colon cancer cells at various concentrations, the ability to inhibit the cell proliferation in dose dependent [11]. The eugenol has a anti-metastatic effects affected cell migration against HeLa cell line treated with various concentration [35]. The cytotoxic potential of Cloves extract on MCF-7 cell lines inhibited to cell proliferation was observed after 24h and 48h of

incubation. The 48h of treatment to inhibition more number of cell growth an achieved lowest IC_{50} values that compared to 24h of treatments [36]. The *S. aromaticum* various solvent extract against HCT human colon carcinoma cells moreover inhibited highest values of cell proliferation respectively [37].

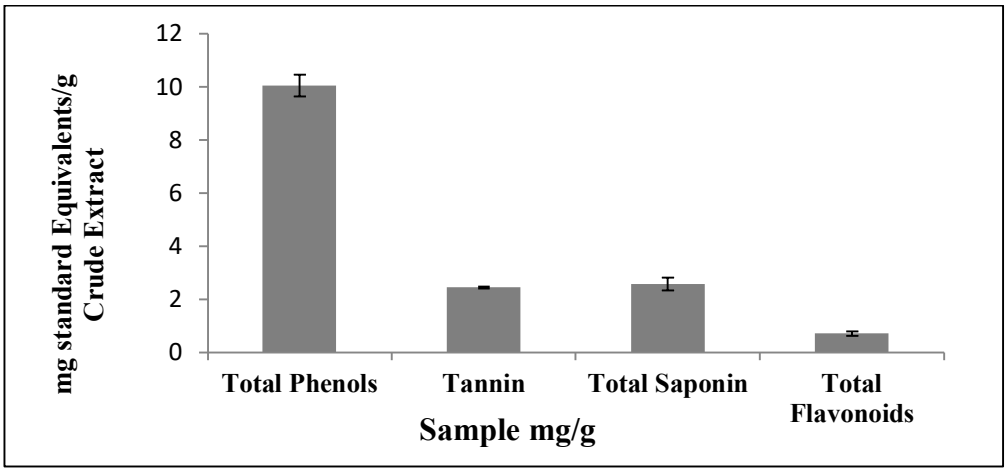

Figure 1. Quantification of primary phytochemicals from flower bud methanolic extracts of *S. aromaticum*

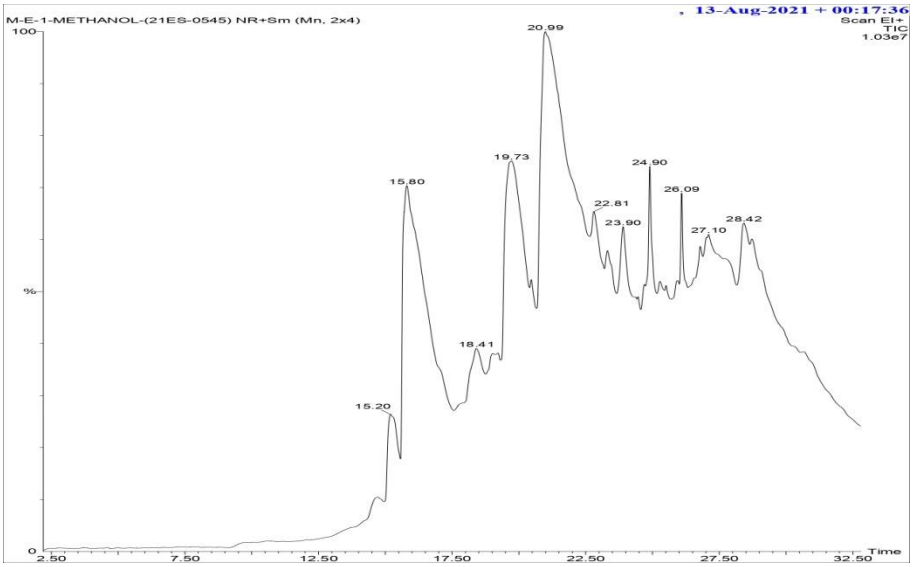

Figure 2. GC-MS chromatogram of flower bud methanolic extracts of *S. aromaticum*

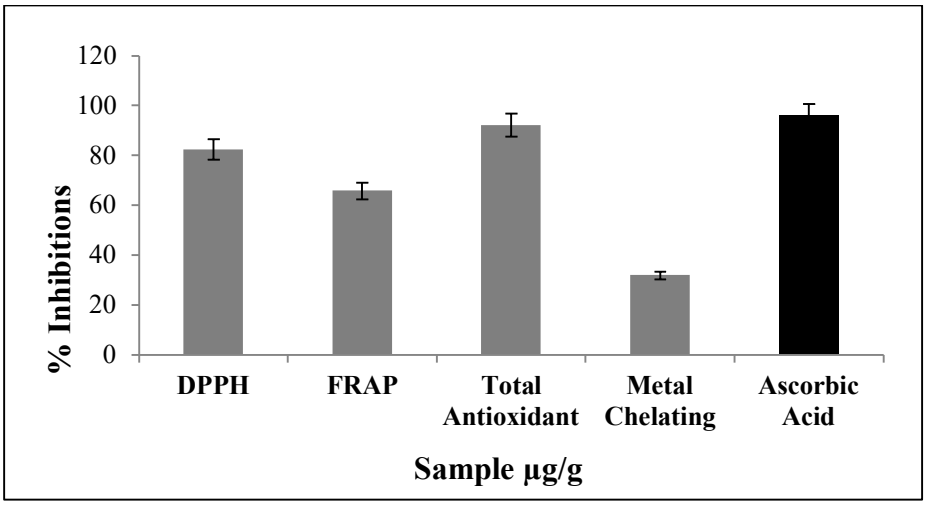

Figure 3. Antioxidant activities of flower bud methanolic extracts of *S. aromaticum*

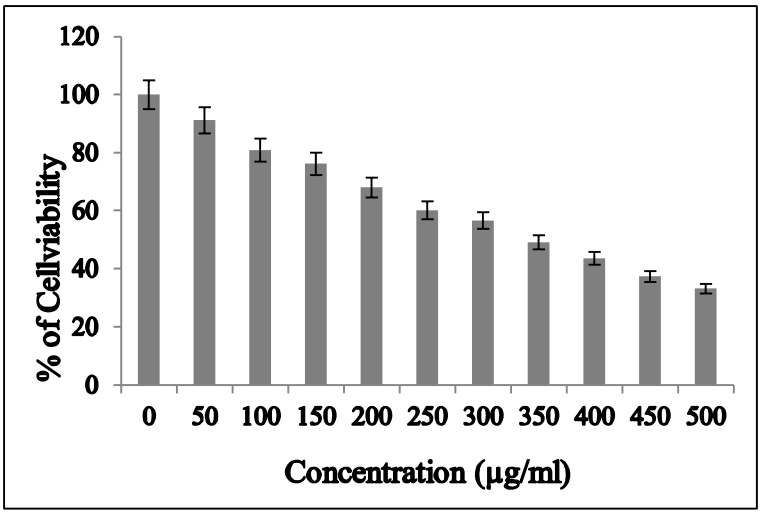

Figure 4. Cytotoxicity effect of various concentrations of flower bud methonilc extracts of *S. aromaticum*

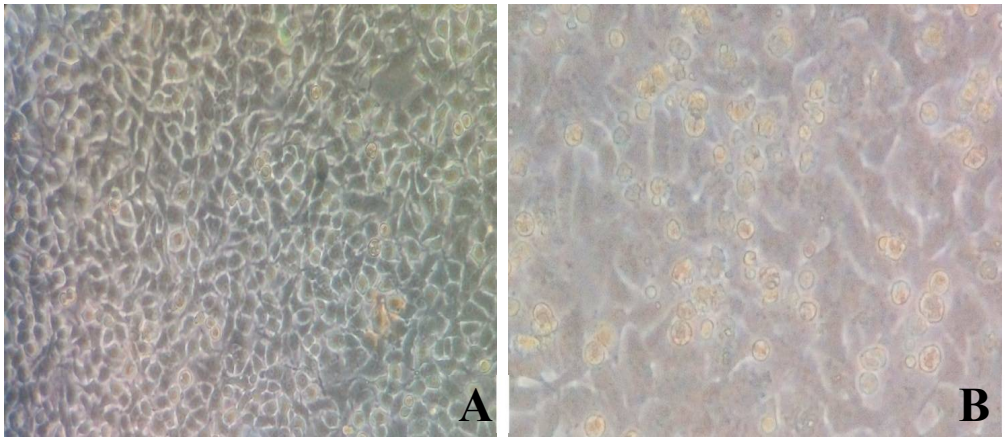

Figure 5. Cytotoxicity of flower bud methanolic extract of *S. aromaticum* was determined after 24 h of treatment. (A) control cells (B) extract treated cells

Table 1. Secondary Phytocompounds from the methanolic extracts of *S. aromaticum*

S.No	RT	Name of the compound	Molecular Formula	MW	Peak Area%
1	15.21	5-phenyl-thiazolidine	$C_9H_{11}NS$	165	2.658
2	15.79	3,6-dimethyl-2-nitrobenzaldehyde	$C_9H_9O_3N$	179	21.96
3	18.42	3-hexyn-2-ol, 5-methyl	$C_7H_{12}O$	112	2.665
4	19.24	cyclopenta[c]furo[3',2':4,5]furo[2,3-h][1]benzopyran-11(1h)-one,	$C_{17}H_{14}O_7$	330	1.430
5	19.75	n-decanoic acid	$C_{10}H_{20}O_2$	172	13.37
6	20.46	3,4-anhydro-d-galactosan	$C_6H_8O_4$	144	1.808
7	20.98	3-decyn-2-ol	$C_{10}H_{18}O$	154	32.09
8	22.82	2,3-epoxyhexanol	$C_6H_{12}O_2$	116	3.917
9	23.91	bicyclo[2.2.1]heptan-2-one, 4,7,7-trimethyl-, semicarbazone	$C_{11}H_{19}ON_3$	209	2.660
10	24.89	hexatriacontane	$C_{36}H_{74}$	506	2.118
11	26.08	1-iodo-2-methylundecane	$C_{12}H_{25}I$	296	1.372
12	26.77	spiro[androst-5-ene-17,1'-cyclobutan]-2'-one, 3-hydroxy	$C_{22}H_{32}O_2$	328	1.445
13	27.09	2-aminononadecane	$C_{19}H_{41}N$	283	5.286
14	28.37	2-methyl-6-methylene-octa-1,7-dien-3-ol	$C_{10}H_{16}O$	152	5.059

CONCLUSION

The results were determined that the *S. aromaticum* flower bud methanolic extracts has high total phenols, which care for cells and tissues against oxidative stress and have the highest radical scavenging activities. The plant flower bud extracts showed a good level of anticancer activity against the A549 lung cancer cell line. As a result, it is concluded that *S. aromaticum* flower buds have extremely high medicinal potential and can be used in further research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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