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Research Article

Antioxidant And Antiproliferative Activity of Thunbergia Fragrans Plant Extracts, Fucoidan and Both Mixture Against Hela Cervical Cancer Cell Lines.

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Abstract
Since ancient times, Thunbergia fragrans have been used as a medicinal herb to treat various chronic abnormalities. We examined the chemical components of T. Fragrans's aqueous extract using established techniques and created a mixture of T. Fragrans and Fucoidan. The chemical components of T. Fragrans's aqueous extract underwent quantitative and qualitative analysis. Additionally, we evaluated the antioxidant and anticancer qualities of T. Fragrans's aqueous extract, Fucoidan, and T. Fragrans's and Fucoidan mixtures. The findings demonstrated that the plant extract's concentration of antioxidants and phytochemicals was higher. Furthermore, TFM had an IC50 value of 0.38 g/mL, higher than TPE, FUC, and TFM. Compared to control cells, HeLa cells treated with TFM showed clear morphological alterations, suggesting its potential as a cervical cancer anticancer treatment and its ability to eradicate malignant cells.
Keywords: · Thunbergia fragrans, fucoidan, Phytochemicals, Anti-cancer activity

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1. Introduction

Known for its robust and woody nature, the Thunbergia fragrans vine in India can grow to varying heights and widths, contingent on the size of the supporting structure it attaches. The foliage of this climbing plant consists of diminutive, elliptical, and triangular leaves. The margins can either be smooth or possess minuscule serrations. The stems place them oppositely, displaying a subtle, soft green hue. This vine yields solitary, uniflorous, or aggregated flowers measuring 1 1/4 inches long. The summer will bring forth these beautiful and aromatic flowers, ranging in color from blue, lavender, and yellow to white. This plant produces seed capsules that have a spherical shape and resemble beaks. This versatile plant can adorn porches, trees, and trellises [1, 2]. Belonging to the Acanthaceae family, this plant exhibits several biological qualities, including antidiabetic, antipyretic, antidote, and anti-inflammatory actions. Due to its elevated flavonoid content, the plant surpasses other plants in quantity [3,4]. The purpose of this research is the goal of separating the various compounds recovered from T. fragrans leaves using various solvents, including

water, ethyl acetate, hexane, and ethanol. We used standard procedures to conduct quantitative and qualitative analyses of the chemical components. This study's overarching goal was to catalogue the phytoconstituents found in T. fragrans extracts [5,6]. We identified the chemicals in the extracts using qualitative and quantitative phytochemical analysis, along with other methods [7, 8]. We also conducted an assessment on the anticancer and antioxidant properties of the T. fragrans extract.

2. Materials and Methods

Sample collection and Authentication

We harvested the complete Thunbergia fragrans plant in September and October from the Kolli Malai Hills in the Namakkal region of Tamil Nadu, India, at coordinates 11.2485 N and 78.3387 E. The Botanical Survey of India (BSI) in Coimbatore has identified and verified specimens of plants, including Thunbergia fragrans, a member of the Acanthaceae family.

2.1.1 Seaweed sample collection

We gathered Sargassum swartzii seaweed from Mandapam, Gulf of Mannar, Tamil Nadu, India. We washed the seaweed in seawater and distilled water to remove debris, then air-dried it before chopping and grinding it in a mixer grinder.

2.1.2 Sample extraction of T. fragrans

The entire T. fragrans plant was cleaned and rinsed We cleaned and rinsed the entire T. fragrans plant under running water, then dried it completely in the shade on a tray at a temperature not exceeding 40 degrees Celsius. After drying, we mechanically crushed the crude drug into a uniform powder, passed it through a 60-mesh filter, and stored it at room temperature until extraction. A Soxhlet device extracted the plant sample, and a rotary evaporator generally evaporated the solvent before extracting the extracted component. The thimble often contains and discards the insoluble component of the extracted substance [8].

2.1.3 Extraction of fucoidan

Fucoidan extraction followed a modified protocol. Initially, 100 grams of the S. swartzii sample underwent ethanol treatment for 12 hours to eliminate proteins and pigments. After centrifugation, the residue was dried. We then extracted five grams of biomass for three hours in 100 mL of acidic water (pH 2.0) at 90°C, repeating this process twice before combining. We centrifuged the combined extracts to precipitate alginic acid, then combined the supernatant with 1% CaCl2. The mixture was then cooled overnight. After another centrifugation, we achieved a 30% concentration of ethanol and cooled the mixture for four hours. We used centrifugation to collect the pellet from the resultant solution. Finally, we added enough ethanol to make the solution 70% concentrated and allowed it to cool overnight [9].

2.2 Phytochemical analysis of an ethanolic extract of Thunbergia fragrans.

We quantitatively and qualitatively identified the phytochemical components from the dried plant extract.

2.3 Qualitative screening of Thunbergia fragrans extract and fucoidan.

Following the standard selection approach outlined by different writers, the ethanolic extract of Thunbergia fragrans and fucoidan was subjected to qualitative analysis [10].

2.3.1 Amino acid

Add two drops of Mayer's reagent to a small amount of plant extracts along the test tube's edge. To identify alkaloids, look for a white or creamy precipitate [11].

2.3.2 Alkaloids

We thoroughly stirred a 2 ml sample with two drops of Wagner reagent. A reddish-purple appearance indicates the presence of alkaloids.

2.3.3 Carbohydrates

Stir the test sample until it dissolves by adding it to the water-filled test tube and shaking it. Incorporate two to three drops of the Molisch reagent. Drop by, slow down the test tube's sides, and add concentrated sulfuric acid. We can detect carbohydrates by creating a purple ring [12].

2.3.4 Flavonoids,

We then mixed the mixture with two or three drops of sodium hydroxide per milliliter (mL). Gradual color loss indicated the presence of flavonoids. After adding a few drops of diluted hydrochloric acid, the initial bright yellow color changed to a pale-yellow color [13].

2.3.5 Glycosides

We mixed two mL of the sample with half a milliliter of ferric chloride (two or three drops) and a solution of glacial acetic acid. We applied concentrated H2SO4, about one milliliter, perpendicular to the test tube's sides [14].

2.3.6 **Phenols,**

We mixed 2 ml of the material with a minimal amount of 10 percent vanillin in ethyl alcohol, then added concentrated hydrochloric acid. The presence of phenols is indicated by their red appearance in the shade [15].

2.3.7 **Protein,**

One milliliter of the sample produced a violet or red color upon adding a few drops of 10% sodium hydroxide and two drops of 3% copper sulfate, indicating the presence of protein [16].

2.3.8 **Saponins,**

We added a small amount of NaCl solution to a test tube that held 5 mL of the sample. We allowed it to tremble with intensity for a brief period before relaxing it. To determine the presence of saponins, we used foam development [17].

2.3.9 Steroids

After shaking the test sample in hydrocarbons and adding a concentration of H2SO4 to the test tube walls, a crimson color emerged, signifying the presence of steroids [18].

2.3.10 **Terpenoids.**

After good mixing, one milliliter of concentrated H_2SO_4 was added to two mL of sample. When terpenoids are present, a reddish-brown tint appears [19].

2.4 Qualitative screening of Thunbergia fragrans

We followed the Harborne (1998) and Khanal (2021) method for the qualitative examination of the ethanolic plant extract of Thunbergia fragrans [20, 21].

2.4.1 Test for Flavonoids:

We combined five grams of powdered material with 100 mL of water and 2 mL of hydrochloric acid (HCl)

solution. The mixture was then cooled and filtered using Whatman No. 1 filter paper after boiling for 30 minutes. After discarding the liquid solution, the residual items were placed on We set the oven to 60 degrees Celsius and removed the filter paper after 30 minutes. We used the following formula to calculate the total flavonoids in the sample.

Percentage of flavonoids = (W2-W1)/W1 X100

To calculate W2, we must add the weight of the paper and falconoid extract to the mass of the disposable filter sheet, with W1 being the weight of the empty filter paper [22].

2.4.2 Test for Alkaloids:

We combined five grams of ground material with 100 mL of 10% acetic acid and thoroughly mixed it. We filtered the mixture with Whatman No. 1 filter paper after letting it sit for four hours. A magnetic stirrer reduced the aqueous extract to 25% of its initial volume through evaporation. After that, small amounts of concentrated ammonium hydroxide (NH_4OH) were added to make the alkaloid content settle out. Subsequently, the solution underwent a second filtration and was rinsed with a 1% NH_4OH solution. We dried the residual material on filter paper by heating it to 60 degrees Celsius for 30 minutes, allowing it to cool, and then weighing it [23].

Alkaloid (%) = (W2-W1)/W1 X100

W2 is the weight of the paper plus the alkaloid extract, while W1 is the weight of the empty filter paper.

2.4.3 Test for Terpenoids:

We combined ten grams of powdered material with 90 mL of ethanol. We filtered the mixture, added 10 mL of petroleum ether, and then repeated the filtration. We quantified the terpenoids using the following formula:

Terpenoid (%) = (Wi-Wf)/Wi X100

The formula is: W_i = weight of the initial plant extracts and W_f = weight of the extracts after drying [24].

2.4.4 Test for Saponins:

We mixed 25-100 mL of 50% alcohol with mL of extract during a 30-minute heating process. We then filtered the solution using Whatman filter paper No. 1. We added 1 gram of charcoal, heated the filtrate to boiling, and then filtered it again. After chilling the mixture, we added an equal volume of acetone to fully precipitate the saponins. We used the following formula to determine the produced saponin concentration [25]:

Saponins (%) = (W2-W1)/W1 X100

The weight of the empty filter paper is W1, while the weight of the paper with saponins and precipitins is W2.

2.4.5 Phenols

We use the spectrophotometer method to quantify the number of phenols. The plant sample is boiled for 15 minutes in a solution containing 50 mL of diethyl ether $(CH_3CH_2)_2O$. The next step is to add 10 mL of distilled water to 5 mL of the heated sample in a 50-mL flask. After adding distilled water, add two mL of NH4OH solution and five mL of concentrated CH3(CH2)3CH2OH to the mixture. To facilitate color development, prepare the sample according to the required standard and leave it to react for 30 minutes. The next step involves measuring the sample at 505 nm using a spectrophotometer [26].

2.4.6 Steroids

We put 1 mL of a steroid test extract solution into volumetric flasks with a capacity of 10 mL. We added two mL of 4N sulfuric acid, mixed 2 mL of iron (III) chloride solution with acid (0.5 milliliter total volume), and then diluted the mixture to 0.5 mL using a potassium hexacyanoferrate (III) solution with a concentration of 0.5% w/v. We heated the solution in a water bath for 30 minutes at a temperature between 70 and 20 C. We diluted the solution with distilled water and stirred it intermittently until it reached the required volume. We used a 780 nm wavelength to measure the absorbance in comparison to the reagent blank [27].

2.5 Characterization of phytochemical compound of *Thunbergia fragrans*

FTIR analysis of Thunbergia fragrans.

We subjected the plant extract of Thunbergia fragrans to FTIR analysis to identify the specific functional groups responsible for its antioxidant activity. To summarize, 0.4 g of total weight was obtained by combining 0.02 g of dehydrated materials with 0.20 g of KBr. Then, using a Fourier-transform infrared spectrometer (FTIR spectra (system 2000) USA, made by Perkin Elmer), infrared spectra were collected over a range of 400–4000/cm. The source for this information is [29].

2.6 Antioxidant activity of ethanolic plant extract of *Thunbergia fragrans and fucoidan.*

2.6.1 22,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

We conducted the experiment using a variant of the Blois test. As stated by Blouss in 1958. We mixed a 1 mL volume of a 0.1 millimolar DPPH methanolic solution with 2 mL of water-based extracts at concentrations ranging from 20 to 100 micrograms per mL. We then left the combination undisturbed for half an hour at room temperature. We combined 1 mL of DPPH solution with 2 liters of distilled water. The spectrophotometer measured the absorbance at 517 nm using a blank as a reference. The reaction mixture's decreased absorbance suggests that the scavenging action for DPPH free radicals has risen. We used ascorbic acid as the reference substance. The samples underwent three rounds of production and measurement [30]. We determined the number of radicals scavenged by each extract by computing the DPPH % inhibition (I%) using the following formula: To find the percentage change, use the formula

Assay of DPPH (I%) = (A0-As)/Ao X100

We denote the control absorption as Ao and the tested extract solution's absorption as.

2.6.2 Ferric Reducing Power Assay (FRAP)

Benzie and Strain (1996) followed the process exactly (Benzie & Strain, 1996). With concentrations ranging from 20 to 100 µg/mLWe combined the FRAP reagent with the aqueous extracts at a volume of 2.3 mL. After that, the mixture was left to incubate at 37ºC for half an hour without exposure to light. Spectrophotometer readings were taken at 593 nm to compare the sample to a control solution that had no sample. All the compounds were present in the sample, with the exception of one. A rise in the absorbance of the reaction mixture indicates an increase in reduction capacity. We measured each sample three times. An ascorbic acid standard was used.

In order to get a standard curve for the ascorbic acid solution, we used the same method that yielded the regression formula. One milliliter of extract yielded milligrams of ascorbic acid equivalents (AAE) [31].

2.6.3 ABTS Assay

The modification of Using the approach of Re et al., carry out the experiment. We prepared a solution of ABTS+ by mixing a water-based mixture of potassium persulfate (2.45 mM) with an ABTS aqueous solution (7 mM) in equal proportions. Before use, we let the final mixture rest for 12–16 hours at ambient temperature and in darkness. The concentrated solution of ABTSThe active solution was prepared by diluting + with methanol until it had an absorbance of 0.70 ± 0.02 at a wavelength of 734 nm. Next, we mixed a 2-milliliter volume of ABTS+ solution with 1 milliliter of the water-based extracts, varying the concentrations from 20 to 80 micrograms per milliliter. We then allowed everything to cool to room temperature for exactly 10 minutes without light. The control group received a mixture of 2.0 mL of ABTS+ solution and 1 mL of double distilled water. We used a spectrophotometer to measure the absorbance at 734 nm in comparison to a blank. As a benchmark, BHT was utilized. We manufactured and measured the samples three times [32]. By applying the following formula, we were able to determine the percentage inhibition (in percentage terms) of the ABTS scavenging activity of each extract:

Assay of ABTS = (A0-As)/Ao X100

The formula provides the percentage transformation of a variable. We denote the control absorption as Ao and the tested extract solution's absorption as.

2.7 *In-vitro* cytotoxic activity

The current study utilized human HeLa cells, procured from the National Centre for Molecular Biology (NCCS) in Pune, India. An incubator maintained the specimens at a regulated temperature of 37 degrees Celsius and a concentration of 5% carbon dioxide. We added an antimitotic solution, 1% antibiotic, 10% fetal bovine serum, and Dulbecco's Modified Eagle's Medium (DMEM) to the cell culture additives. We seeded 1,104 HeLa cells per well in 96-well plates. We then exposed them to serum-free media and various quantities (5, 10, 25, 50, 75, 100, 150, and 200 g/mL) of Thunbergia fragrans extract for a full day. Once the treatment period ended, we removed the cells from the old media. The next step involved adding MTT to 1X PBS at a concentration of 0.5 mg/mL, introducing the mixture into a CO2 incubator, and maintaining the temperature at 37°C for four consecutive blocks. After the incubation period, we used 200 microliters of PBS to purify the cells and remove the MTT-containing medium from the culture medium. The formazan dye exhibited a violet-blue coloration upon dissolution of the crystallized substance in 100 liters of DMSO. We used a spectrophotometer to measure the absorption at 570 nm [33].

2.8 Statistical analysis

We used the statistical software STATGRAPHICS Centurion XV to evaluate the data, comparing the means of each treatment through an ANOVA test. We used the LSD test ($p < 0.05$) to find out if there were significant variations in the parameter means. The group determinations' results are shown as the mean ± standard deviation (STD).

3. Results and Discussion

3.1 Phytochemical analysis of an ethanolic extract of *Thunbergia fragrans*

3.1.1 Qualitative screening

Table 1 presents the chemical constituent analysis of an ethanolic mixture containing Thunbergia fragrans. There were no glycosides or saponins in the extract, but it did include compound families that include alkaloids, flavonoids, steroids, triterpenes, phenols, thiols, anthraquinones, volatile oils, and tannins. The polar nature of the biochemicals in the extract likely leads to their dissolution in the ethanol extract. Moreover, studies have demonstrated that ethanol extract can permeate cell membranes and enhance the rapid physiological assimilation of plant constituents [34].

Table 1: Qualitative screening of phytochemicals for the plant extract of Thunbergia fragrans, fucoidan and mixed concentration

S.No	Phytochemicals	TPE	FUC	TFM
1	Amino acid	-	-	-
2	Alkaloids	++	-	++
3	Carbohydrates	+	+	+
4	Flavonoids	+	+	+
5	Glycosides	+	-	-
6	Phenols	-	-	-
7	Protein	+	-	+
8	Saponins	-	-	-
9	Steroids	++	-	++
10	Terpinoids	+	+	+

3.1.2 Quantitative screening

A Table 2 displays the results of a quantitative analysis of the phytochemicals found in Thunbergia fragrans's ethanolic extract. Phytochemicals found most frequently in the ethanolic extract are tannins, alkaloids, and phenols due to their high concentrations. The amounts of flavonoids, on the other hand, are lower. Phenols may have powerful anti-cancer, anti-atherosclerosis, and anti-aging degenerative brain disease effects due to their pharmacological properties [35]. In addition, research has demonstrated that the plant extract, which is rich in

phenolic chemicals, has a crucial function in metabolic activity and the treatment of chronic diseases.

Table 2: Quantitative screening of phytochemicals

S. No	Test	Concentrations	(µg/g)
1	Alkaloid	50	146 ±0.21
		100	132 ± 0.25
2	Flavonoids	50	112 ± 0.14
		100	130 ± 0.23
3	Tannins	50	162 ± 0.32
		100	154 ± 0.37
4	Terpenoids	50	146 ± 0.23
		100	110 ± 0.18
5	Phenols	50	124 ± 0.20
		100	120 ± 0.19

3.2 Characterization of phytochemical compound

We analyzed the active phytochemical components of the Thunbergiafragrans plant extract using Fourier transform infrared spectroscopy (FTIR). Figure 1 and Table 3 display the outcomes. The ethanolic extract of Thunbergia fragrans has alkynes, phenols, alcohols, aliphatic alkanes, aliphatic amines, and alkyl groups. It also has aromatic chemicals in it. We performed FTIR analysis to identify potential interfacial groups between the plant extract and the capping agents. In the investigation, the researchers detected strong absorption at 3631.65, 3438.36, 2924.01, 2854.40, 1738.69, 1627.99, 1461.96, 1382.32, 1320.65, 1205.18, 1164.62, 1120.46, 1088.08, and 1022.38 cm^{-1}. The presence of bands at 3631.65 and 3596.32 cm^{-1} in the FTIR spectrum is indicative, as is the free O-H stretch, respectively. The primary and secondary amines, amides, and N-H stretch were detected at 3438.36 cm^{-1}. The groups present at 2924.01 and 2854.40 cm confirmed the stretch of C-H alkanes. At 1738.69 cm, we detected a C=O aldehyde band. We found C=C alkenes at 1627.99 cm, -CH- (bend) at 1461.96 cm, C-X fluorides stretch at 1382.32 cm, and C-N amino acids at 1320.65 cm. The bands at 1205.18, 1164.62, 1120.46, and 1088.08 cm determined the C-O primary, secondary, or tertiary structure of an alcohol. The C-N sThe band at 1022.38 cm found the C-N stretching vibration of amines. ing provides more evidence that biological molecules may have a role in the production and stabilization of extracts via proteins' free amine groups [36].

Table 3: FTIR characterization of the plant extract of *Thunbergia fragrans*

S.No	Peak Band (cm^{-1})	Vibration Type	Functional groups	Intensity
1	3336	O-H stretching	Alcohol	Strong
2	2958	C-H stretching	Alkane	Medium
3	2893	O-H stretching	Alcohol	Weak
4	1661	C=C stretching	Alkene	Medium
5	1382	C-H stretching	Aldehyde	Medium
6	1103	C-O stretching	2 º Alcohol	Strong
7	1045	CO-O-CO stretching	Anhydrite	Strong
8	873	C-H Bending	1 3-disubstituted	Strong
9	635	C-Br Stretching	Halo compound	Strong

3.3 Antioxidant activity of *Thunbergia fragrans* and fucoidan and mixed concentration

3.3.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The graph (Fig. 1) demonstrates that 100 µg/mL is the concentration; the combination of TFM exhibited the highest percentage of scavenging activity at 47.28±12.66. TPE followed this at 20.76±3.723 and FUC at 40.12±9.2. There was a notable disparity ($p < 0.05$) in the concentrations of TPE, FUC, and TFM. The IC50 value of TFM is 8.0 µg/mL, followed by FUC and TPE at 5.8 and 2.3 µg/mL, respectively [37].

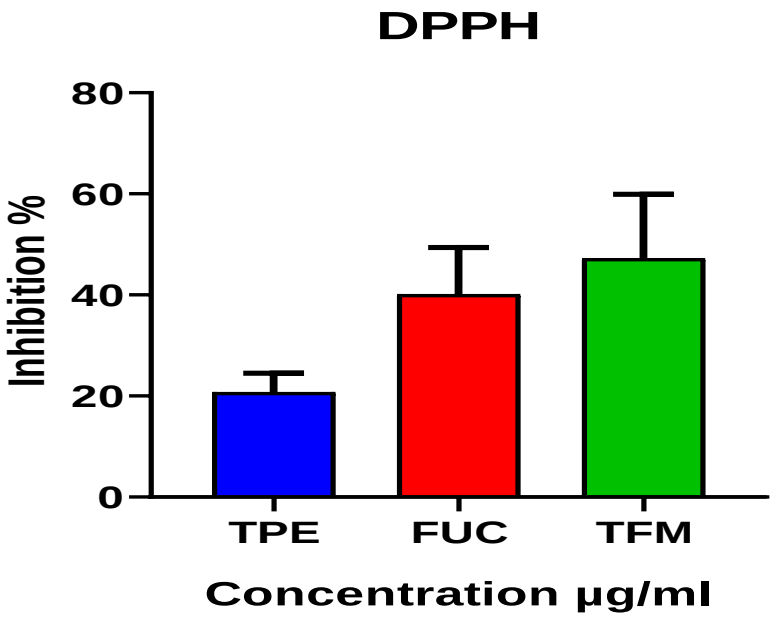


Figure: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay method for Thunbergia fragrans Plant extracts, fucoidan and both mixture

3.1.2 Ferric Reducing Power Assay (FRAP)

As shown in Figure 3As an example, the water-based extracts of as an example, the water-based extracts of TPE, FUC, and TFM the amount of ascorbic acid equivalents (AAE) per millilitre of extract is given. In terms of reducing ability, TFM was the most effective of all the drugs that were evaluated. There was a notable disparity ($p<0.05$) in on all TPE, FUC, and TFM concentrations. The next two compounds, TPE and FUC, were detected after TFM respectively, with IC50 values of 0.06 and 0.02 µg/mL.

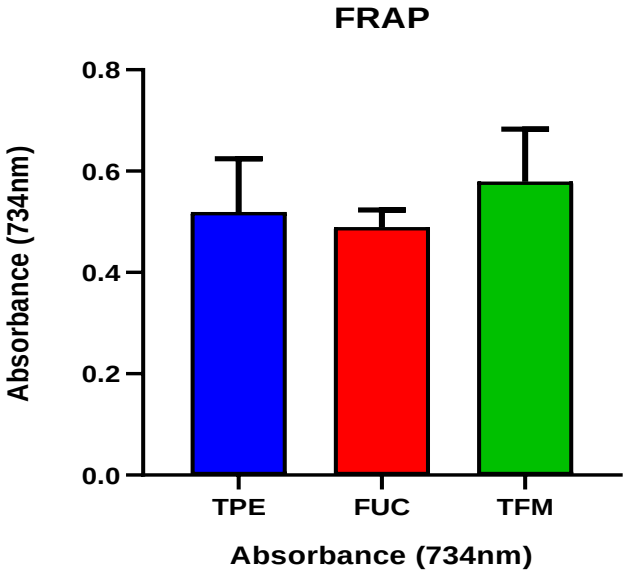


Figure 2: Ferric Reducing Power Assa for Thunbergia fragrans Plant extracts, fucoidan and both mixture

3.1.3 ABTS Assay

The graph (Fig. 3) demonstrates that 100 micrograms per mL, the combination of TFM, exhibited the highest percentage of scavenging activity at 52.44±9.737. TPE followed this at 46.19±8.803 and FUC at 32.18±8.540. There is a notable distinction ($p < 0.05$) between all TPE, FUC, and TFM concentrations that were observed. The IC50 value of TFM is 6.0 µg/mL, followed by FUC and TPE at 5.5 and 5.3 µg/mL, respectively.

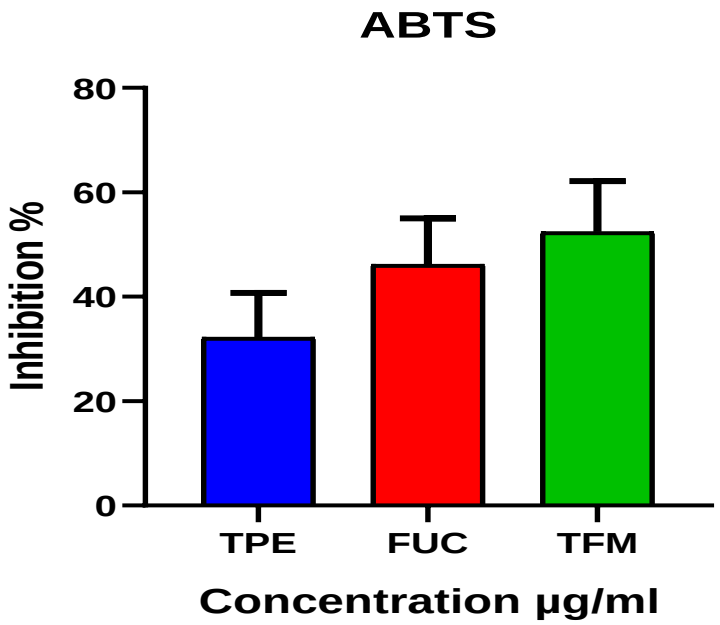


Figure 3: ABTS Assay for Thunbergia fragrans Plant extracts, fucoidan and both mixture

3.2 Invitro cytotoxic activity by MTT assay

We used TPE, FUC, and TFM for the in vitro cultivation of HeLa cells. We evaluated their cytotoxic properties using the MTT assay. We administered different doses of TPE, FUC, and TFM to the cells, using untreated cells as a control. The perWe assessed the percentage of viable cells after exposing them to various doses of TPE, FUC, and TFM (20, 40, 60, 80, and 100 g/mL). experiment revealed a decrease in cell viability as the concentration of TFM rose. Figure 4 displays the IC50 value of 93.63 µg/mL. The control cells revealed a distinct morphology in the cervical cancer cells treated with TFM, indicating their potential to eliminate cancer cells and serve as cervical cancer anticancer treatments. The MTT experiment revealed a decrease in cell viability as the concentration of TFM rose. Figure 4 displays the IC50 value of 93.63 µg/mL. The cervical cancer cells treated with TFM exhibited distinct morphology compared to the healthy cells, indicating that it has the potential to kill cancer cells and be used as a cervical cancer treatment [38].

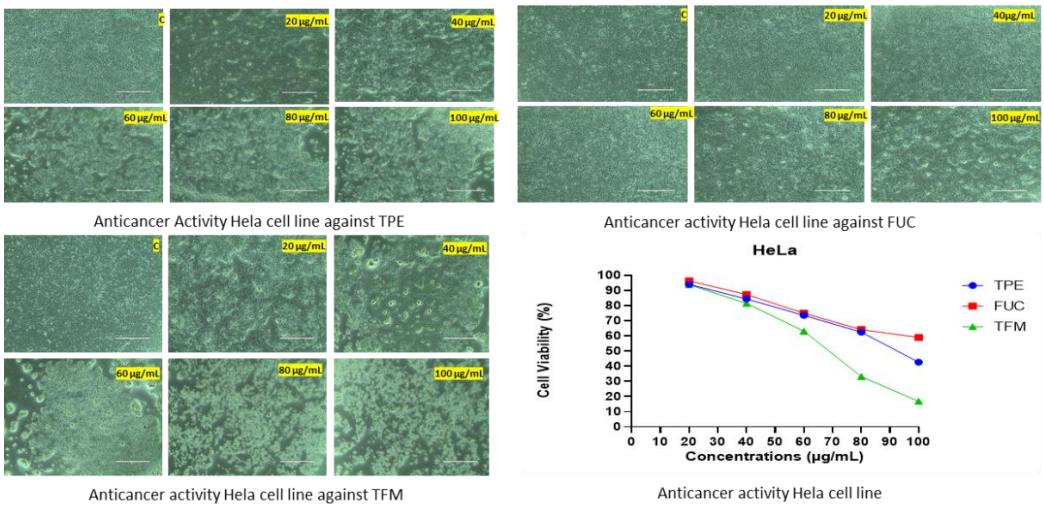


Figure 4: Invitro cytotoxicity studies of Thunbergia fragrans Plant extracts, fucoidan and both mixture

5 CONCLUSION

Since ancient times, people have used Thunbergia fragrans to treat a variety of persistent illnesses. Clinically advantageous components such as vitamins and flavonoids can mitigate the adverse effects of pharmaceutical medications. Our studies established the presence and function of these biological substances. Furthermore, our research has demonstrated the beneficial effects of Thunbergia fragrans in combating free radicals. We should evaluate the sample's antioxidant activity concurrently using various methods, as distinct processes may yield divergent outcomes. The investigation revealed that the Thunbergia fragrans extract included a higher concentration of

phytochemicals and antioxidants. We observe different peaks in the Fourier transform infrared (FTIR) spectra for aromatic chemicals, alkenes, alcohols, alkanes, and aliphatic amines. The ethanolic extract of Thunbergia fragrans can slow the growth of cancer cells by stopping them from using the products of biochemical reactions that they create. We plan to conduct a follow-up investigation to analyze the inhibitory effects of Thunbergia fragrans ethanolic extract on cancer cell proliferation across various expression systems. According to our research, the biochemical parts that we got from Thunbergia fragrans are very important for maintaining homeostasis and could be used as antioxidants and cancer fighters.

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