Phytochemical Screening Of Methanolic Extract Of *Vanda Tessellata* Fraction 2 And 3 On Cell Cycle Distribution Of Colorectal Adenocarcinoma Cell Lines

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ABSTRACT
Roughly 90% of malignant large bowel tumors are colorectal adenocarcinomas. Early diagnosis via screening programs is crucial since early-stage colorectal cancer often exhibits no symptoms. This research evaluated the phytochemical screening of methanolic extract of *Vanda tessellata* on cell cycle distribution of colorectal adenocarcinoma cell lines. The mature and fresh leaves of *Vanda tessellate* were collected from the Dehradun, Uttarakhand in the month of October. Leaves were identified and authenticated by the botanist at CSIR-National Botanical Research Institute (NBRI), Lucknow, India. The leaves of *Vanda tessellata* were extracted using petroleum ether, DCM and methanol solvent. Phytochemical screening was performed for all the 3 extracts. Isolation of the compounds was performed using column chromatography. *In-vitro* anti-cancer activity of extracts was evaluated through inhibition assay using Colo 205 cell line, HT-29 cell line, and INT407 cell line. It concluded that various fractions' IC50 concentrations on Colo205 and HT-29 were either equal to or higher than the methanolic extract's IC50 concentration. This could indicate that the extract's specific cytotoxicity against adenocarcinoma cells may be due to the combined effects of many chemical constituents.

Keywords: *Vanda tessellata*, anti-tumor, phytochemicals, adenocarcinoma, cell cycle.

INTRODUCTION

Adenocarcinomas are a kind of malignant tumor that can develop in a number of body areas. Neoplasia of epithelial tissue with glandular origin, glandular features, or both is what is meant by this term. While adenocarcinomas are a subset of carcinomas, they are also occasionally referred to by more specific terminology that do not include the word [1]. The most prevalent type of breast cancer, invasive ductal carcinoma, is adenocarcinoma, but it does not use the term in its name. In contrast, esophageal adenocarcinoma uses it to differentiate it from esophageal squamous cell carcinoma, another common type of esophageal cancer [2]. Adenocarcinomas account for the most common type of colorectal cancer. The colon has several glands, which explains this. Normal colonic glands are typically tubular and simple, containing a combination of water-absorbing and mucus-secreting goblet cells. These glands lubricate the feces as they travel to the rectum by secreting mucus into the colon's lumen [3]. The progression of these glands from benign to invasive, malignant colon cancer is predicted when they experience a variety of genetic alterations [4].

Vanda tessellata requires direct sunlight exposure. It is mostly found in deciduous woodlands that range from damp to dry. Fruiting occurs all year round, mostly from June to January. Perianths may be yellow, green, brown, or gray, depending on the floral design and color variation [5]. The lips are either bluish-purple, pink, or purple. It has antibacterial, cytotoxic, and antioxidant properties [6]. According to reports, *V. tessellate* contains a number of bioactive substances, including phenols, tannins, alkaloids, flavonoids, terpenoids, glycosides, steroids, glucoside, tannins, β- and γ sitosterol, along with chain aliphatic compounds, fatty oils, and resins [7]. The current research was focused on the the phytochemical screening of methanolic extract of *Vanda tessellata* on cell cycle distribution of colorectal adenocarcinoma cell lines.

MATERIAL AND METHODS

Requirements

Dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), MTT reagent, DCFH-DA, RIPA buffer with protease and phosphatase inhibitors, Petroleum ether (60–80°C), Dichloromethane (DCM) and Methanol. Colo 205 cell line, HT-29 cell line, and INT407 cell line.

Collection and extraction: The mature and fresh leaves of *Vanda tessellate* were collected from the Dehradun, Uttarakhand in the month of October. Leaves were identified and authenticated by the botanist at CSIR-National Botanical Research Institute (NBRI), Lucknow, India.

Extraction of plants

The dried plant material of *Vanda tessellata* roots (500g) was powdered using a mechanical grinder and subjected to Soxhlet extraction. The sequential extraction was carried out using Petroleum ether (60-80) then Dichloromethane (DCM) and finally with Methanol as the solvents for 8 hours. After the extraction process, the solvent was evaporated under reduced pressure using a rotary evaporator to obtain a concentrated extract [8].

Phytochemical Screening [9]

Flavonoids

Alkaline Reagent Test

2ml of the extract was mixed with 2ml of 2% sodium hydroxide solution and shaken. Formation of an intense yellow color, which becomes colorless upon adding dilute hydrochloric acid, confirms the presence of flavonoids.

Alkaloids

Dragendorff's Test

To 2 mL of the extract, 2-3 drops of Dragendorff's reagent (potassium bismuth iodide solution) were added. The formation of an orange or reddish-brown precipitate indicates the presence of alkaloids.

Wagner's Test

To 2 mL of the extract, 2-3 drops of Wagner's reagent (iodine in potassium iodide) were added. The formation of a reddish-brown precipitate confirms the presence of alkaloids.

Glycosides

Legal's Test

2 mL of the extract was treated with 1 mL of pyridine and 1 mL of sodium nitroprusside solution and shaken well. The appearance of a pink or red color indicates the presence of glycosides.

Killer-Kiliani Test *(Specific to Ipomea aquatica)*

2 mL of the extract was mixed with 2 mL of glacial acetic acid containing a drop of ferric chloride, followed by careful addition of concentrated sulfuric acid. The appearance of a reddish-brown ring at the interface and bluish-green coloration in the upper layer confirms glycosides.

Amino Acids

Ninhydrin Test

2 mL of the extract was mixed with 2 mL of 0.25% ninhydrin solution and gently heated in a water bath for 2-3 minutes. The development of a purple or blue color indicates the presence of amino acids.

Tannins

Ferric Chloride Test

2 mL of the extract was treated with 2-3 drops of 5% ferric chloride solution. The appearance of a greenish-black or bluish-black color indicates the presence of tannins.

Vanillin-Hydrochloride Test

2 mL of the extract was mixed with 2 mL of vanillin reagent (vanillin in concentrated HCl). A red or pink color indicates the presence of tannins.

Saponins

Foam Test

2 mL of the extract was diluted with 5 mL of distilled water and shaken vigorously for 2-3 minutes. The test tube was allowed to stand for 10 minutes. Formation of stable and persistent foam indicates the presence of saponins.

Terpenoids

Salkowski Test

2 mL of the extract was mixed with 2 mL of chloroform. Then, 2 mL of concentrated sulfuric acid was carefully added along the sides of the test tube. A reddish-brown coloration at the interface confirms the presence of terpenoids.

Phenols

Ferric Chloride Test

2 mL of the extract was treated with 2-3 drops of 5% ferric chloride solution. The appearance of blue, green, or black coloration indicates the presence of phenols.

Steroids

Salkowski Test

A few drops of extract dissolved in chloroform were mixed with concentrated sulfuric acid and shaken gently. The appearance of a red or reddish-brown color at the interface indicates the presence of steroids.

Isolation of the compounds using column chromatography [10]

- A column was loaded with silica gel (60-120 mesh size) up to approximately 70% of its length. The column was loaded or packed using wet loading method to ensure proper and uniform seating of silica gel in the column without leaving any air bubbles or imperfection, the column was also gently tapped during the packing to ensure proper packing.
- The extract was dissolved in the corresponding solvent and silica gel (3X the weight of extract) was mixed with the solution to allowing appropriate adsorption of the extract on to the silica. Solvent was evaporated until dry adsorbed silica is obtained.
- The adsorbed silica gel was poured on top of the packed column and a piece of cotton was placed on top to prevent the top layer from getting disturbed.
- The column was run from this point on starting with pure petroleum ether gradually increasing polarity to 0.5% chloroform then 1%, 2%, 3%, 5%, 10%, 20%, 50%, 80% and then 100% chloroform then in the same way increasing the polarity with ethyl acetate from 0.5% to 100% and the finally increasing polarity with methanol 0.5%, 1%, 3%, 5% and finally 10%. The chromatography was ceased at this point as the maximum achievable separation was reached.
- Different fractions were collected and the fractions that displayed the same TLC profile were pooled together. The process of purification was repeated for isolated compounds on a smaller column to obtain highly pure compounds.

Cell Culture

The "National Centre for Cell Sciences in Pune, India" provided the colorectal cancer cell line Colo205. "Dr. Ramakrishnan of BARC," situated in Mumbai, India, ENT407, generously donated this item. Colo205 was preserved "in Roswell Park Memorial Institute medium (RPMI)" 1640. "The cell lines were maintained using complete media, pH 7.4 (with 10% heat-inactivated foetal bovine serum added as a supplement), L-glutamine (2 mmol/l), 100 units/ml penicillin, and 100 ug/ml streptomycin. The cultured cells were kept at 37°C in a humidified atmosphere with 95% air and 5% CO2.

Evaluation of i*n-vitro* anti-cancer activity

Inhibition Assay

Preparation of cell culture

Seed 100µl of the cell suspension (5×10^4 cells/well) into a 96-well plate and incubate for 24 hours at 37°C with 5% CO_2 to allow for cell attachment.

Preparation of Extract solution

Plant extract of *Vanda tessellata* was dissolved in DMSO to prepare a stock solution (10 mg/ml). Prepare serial dilutions of the stock solution in a complete medium to obtain various concentrations. Ensure the final DMSO concentration does not exceed 0.1% in each well to avoid cytotoxic effects.

Assay

Replace the medium in the wells with 100 µL of medium containing the respective extract concentrations. Include a control group with untreated cells (medium only) and a vehicle control with 0.1% DMSO. Incubate the plate for 24–48 hours at 37°C with 5% CO_2. After incubation, add 10 µL of MTT solution (5 mg/mL in PBS) to each well. Incubate the plate for 4 hours at 37°C in the dark. During this period, the viable cells metabolize MTT to formazan crystals. Carefully remove the medium from the wells without disturbing the

crystals. Add 100 µL of DMSO to each well to dissolve the formazan crystals. Gently shake the plate for 10 minutes to ensure complete solubilization [11].
Measure the absorbance at 570 nm using a microplate reader.
Calculate the percentage cell viability using the formula:

$$\text{Cell Viability (\%)} = \left(\frac{\text{Absorbance of Treated Wells}}{\text{Absorbance of Control Wells}}\right) \times 100$$

Determine the IC_{50} value (concentration required to inhibit 50% of cell viability) for each extract. Plot the concentrations of the extract (logarithmic scale) on the x-axis and the cell viability (%) on the y-axis. A sigmoidal curve is typically observed in cytotoxicity assays

Effect on Cell Cycle Progression
Treatment: Treat cancer cell lines with varying concentrations of the DCM extract.
Analysis: Use flow cytometry to analyse cell cycle phases (G0/G1, S, G2/M) post-treatment.
Quantify the population of cells in each phase to determine the extract's effect on cell cycle checkpoints.
Analysis: Employ fluorescence microscopy or flow cytometry to detect phosphatidylserine externalization (indicative of apoptosis).

RESULT AND DISCUSSION

Percentage yield
The below table refers the percentage yield of the plant in different solvent system.

Table 1. Extraction details of plant

Plant Material	Solvent Used	Duration of Extraction	Extraction Method	Final Yield from Solvent (g)
Vanda tessellata	Petroleum Ether (60-80°C)	8 hours	Soxhlet Extraction	50.2 g
	Dichloromethane (DCM)	8 hours	Soxhlet Extraction	42.8 g
	Methanol	8 hours	Soxhlet Extraction	38.20 g

Phytochemicals determination

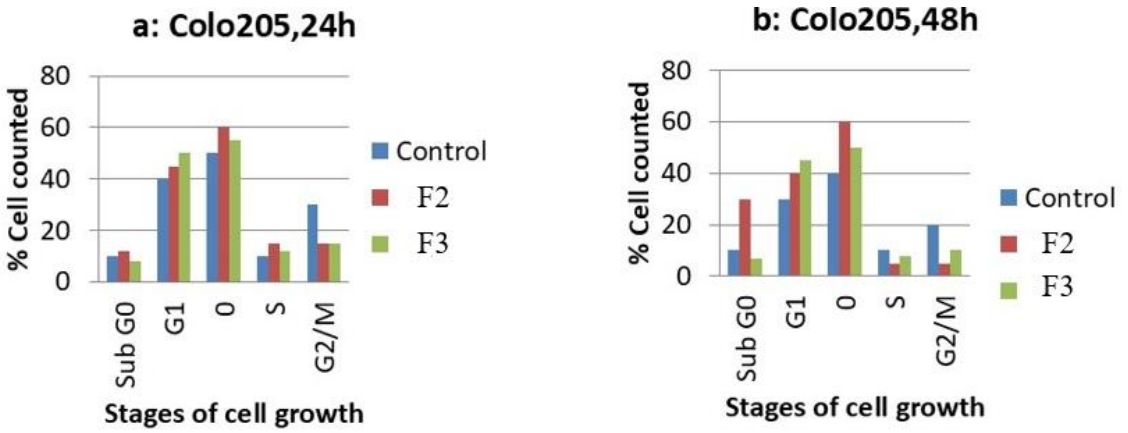
Table 2: Qualitative Tests for *Vanda tessellata* Leaves Extracts

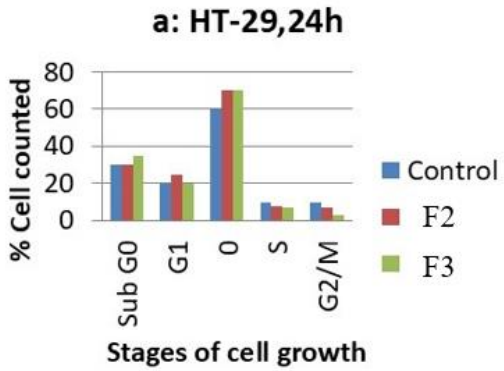
Chemical Class	Test	Extract 1	Extract 2	Extract 3
Flavonoid	Alkaline Reagent	-	+	+
Alkaloids	Dragendroff's Test	-	-	+
	Wagner's Test	-	-	+
Amino Acids	Ninhydrin Test	-	-	-
Tannins	Ferric Chloride Test	-	-	+
	Vanillin Hydrochloride Test	-	-	+
Saponin	Foam Test	+	+	+
Terpenoid	Salkowski Test	+	+	+
Phenols	Ferric Chloride	-	+	+
Steroid	Salkowski Test	+	+	+
Glycosides	Legal's Test	-	+	+
	Killer-Kiliani Test	-	+	+

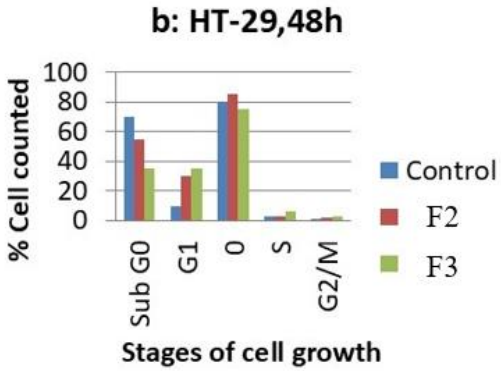
Assessment of anticancer activity of the fraction 2 and 3 on the cell cycle; obtained from the methanolic extract of *Vanda tessellate*
The study investigated the effects of Fractions 2 (F2) and 4 (F4) on cell cycle arrest in Colo205, HT-29, and INT407 cell lines at 24 and 48 hours. The results reveal significant alterations in the cell cycle distribution across different phases, emphasizing the influence of these fractions on cancer cell progression. For the Colo205 cell line, F2 treatment for 24 hours resulted in an increase in the G0 phase population (17.44%) compared to the control (12.17%), while the G1 phase population rose to 45.7%, cumulatively leading to a marked increase in the G0+G1 phase (63.14%). This was accompanied by a decrease in the S (18.86%) and G2/M (13.56%) phases compared to control values. At 48 hours, F2 significantly increased the G0+G1

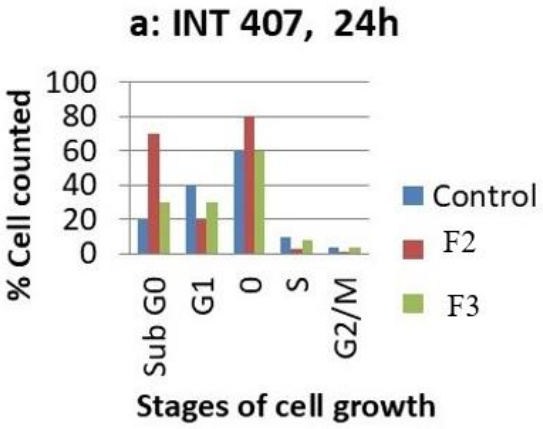
population to 76.73%, with a notable reduction in the S (7.84%) and G2/M (7.28%) phases, suggesting effective cell cycle arrest at G0+G1. F3 also induced similar trends, with increases in the G0+G1 population to 59.31% at 24 hours and 59.69% at 48 hours.

In HT-29 cells, F2 and F3 demonstrated substantial differences in the G0+G1 phase population at both time points. At 24 hours, F2 increased the G0+G1 population to 66.94% compared to the control (58.26%), with a concurrent decrease in the S phase (11.83%). F3 displayed a similar trend, raising the G0+G1 phase to 66.69%. At 48 hours, the G0+G1 population further increased to 85.87% with F2 treatment, while F3 maintained a high level at 74.18%, indicating its sustained effect. The S and G2/M phases exhibited pronounced reductions for both fractions, highlighting their efficacy in blocking cell cycle progression. INT407 cells showed unique dynamics, particularly with F2. At 24 hours, F2 caused a dramatic increase in the G0+G1 population to 91.05% compared to 64.18% in controls, while the S phase plummeted to 3.54%. F3 caused a modest rise in the G0+G1 phase (65.85%) and a slight reduction in the S phase (9.78%). At 48 hours, both F2 and F3 maintained a high G0+G1 phase population (79.84% and 79.19%, respectively), with a minimal S phase presence (10.25% and 11.92%, respectively).









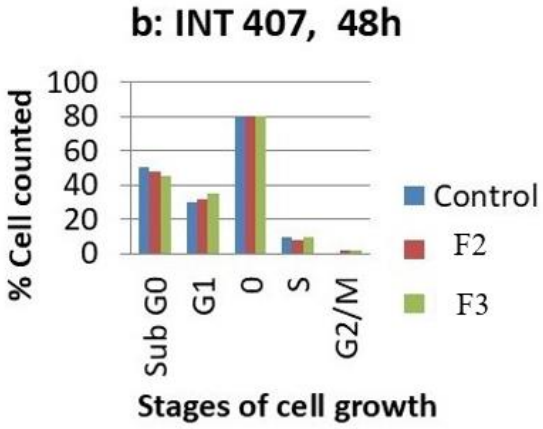


Figure 1. Graphical representation of the anti-cancer effect of fraction 2 and 3 of methanolic extract of *Vanda tessellate* against various cell lines.

Apoptosis eventually occurs in the cells that are stopped in any one stage of the growth cycle [13]. Colo205 and H T-29 are represented in the tables, respectively. There are an estimated nine different types of large intestine cancers, including colorectal adenocarcinomas. Early detection through screening programs is essential since early-stage colorectal cancer is often characterized by the lack of symptoms1. To examine cellular activity, some examples include measuring the effects of tumor necrosis factor-a or -b, assessing cytotoxic or growth-inhibiting chemicals, such as inhibitory antibodies, and measuring macrophage-induced cell death [2].
According to the tables above, all three cell lines exhibit a stoppage of the cell development cycle in the GO/G1 phase upon exposure to the IC50 concentration of fractions 2 and 3. Overall, these findings underline the potency of F2 and F3 in inducing cell cycle arrest, primarily at the G0+G1 phase, across all tested cell lines. F2 demonstrated a stronger influence compared to F3, particularly in INT407 cells. This arrest reflects the potential therapeutic efficacy of these fractions in impairing cancer cell proliferation by halting progression through the cell cycle.

CONCLUSION

Colorectal adenocarcinoma cells are malignant epithelial cells that develop from the glandular epithelial cells lining the colon and rectum. They make up 98% of all colonic cancers and are the most common type of gastrointestinal cancer. Certain separated fractions were shown to be nearly equitoxic to both normal intestinal cells and colorectal cancer cells during the phytoconstituents analysis and column chromatography. It concluded that various fractions' IC50 concentrations on Colo205 and HT-29 were either equal to or higher than the methanolic extract's IC50 concentration. This could indicate that the extract's specific cytotoxicity against adenocarcinoma cells may be due to the combined effects of many chemical constituents.

CONFLICT OF INTEREST

None.

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