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PRELIMINARY PHYTOCHEMICAL SCREENING AND QUALITATIVE ANTICANCER ACTIVITY OF *TECTONA GRANDIS* AND *ANNONA SQUAMOSA* ON HELA CELL LINE

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ABSTRACT: *Tectona grandis* and *Annona squamosa* are two medicinal plants of Lamiaceae and Annonaceae families respectively. Both of them are traditionally used to treat various diseases. As cancer is a life-threatening disease and cervical cancer is one of the most dangerous types of cancer, currently researchers' goal is to investigate natural sources of anticancer agents. The objective of the present research is to investigate the preliminary phytochemicals and qualitative anticancer activity of the ethanolic leaves extracts of these two plants. Standard methods of phytochemical scrrening were employed to identify the secondary metabolites including carbohydrates, glycosides, saponins, steroids, flavonoids, tannins, phenols, coumarins, resins, and alkaloids. Besides, qualitative anticancer activity on HeLa cell line was investigated for these two plants from Centre for Advanced Research in Science (CARS), Dhaka, Bangladesh, using their standard procedure. Preliminary phytochemical analysis revealed the presence of steroids, tannin and alkaloids in the ethanolic extract of leaves of *Tectona grandis* and it is revealed that carbohydrates, glycosides, steroids, tannins, alkaloids, flavonoids, phenolic compounds, and coumarins were present in the extract of *Annona squamosa*. After 48 hours of administration of the extracts, *Tectona grandis* was found to inhibit 95% cell growth of HeLa cells whereas *Annona squamosa* was found to inhibit 80-90% of HeLa cells. As *Tectona grandis* is found to exert more potent anticancer activity, the future prospect of this research is to conduct *in-vivo* assessments, followed by the identification and isolation of the active anticancer agents.

INTRODUCTION: Plants are being used as an important source of medicine throughout human history. In developed countries, approximately 25% of medicinal drugs are derived from plants and their derivatives specifically from phytochemicals which are used as templates for lead optimization for creating safer and more effective medicines.

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Research on natural products is required to identify the medicinal value of plants by investigating the traditional uses, existing scientific evidence and potential therapeutic compounds [1].

Plant extracts are enriched with various secondary phytochemicals which are nonessential for the plant growth. These metabolites including lipids, amino acids, carbohydrates and nucleic acids play an important role that support human health by preventing chronic diseases. Various extraction methods have been used to separate these bioactive components from plants [2]. *Tectona grandis* is a valuable tropical timber species of Lamiaceae family known for its high durability, strength and

natural resistance to decay. It is traditionally used in herbal medicine to treat inflammatory conditions, digestive disorders, respiratory problems and health-related issues due to having medicinal properties [3].

Annona squamosa, belongs to the Annonaceae family, is widely distributed across the tropical and subtropical areas of Asia, Australia, Americas. This species is commonly known as sugar apple, custard apple, or sweetsop. It has many other local names in various countries. It is widely used in traditional medicine. Various parts of the plant, leaves, fruits, seeds and bark, have been employed to treat numerous diseases. Many of its constituents have remained unexplored [4]. The objective of the present study is to investigate the presence of preliminary phytochemicals and the qualitative anticancer properties of the leaves extract of the two plants. Cancer, characterized by uncontrolled abnormal cell proliferation followed by genetic mutations. Among various types cervical cancer is one of the major types that affects cervix, the region connecting the uterus and vagina. It can cause serious complications such as abnormal pain, bleeding and kidney failure followed by the build up of malignant tumor cells on the cervix zone [2]. Due to high the mortality rate there is a need to research the natural sources as anticancer agents. The HeLa cell line obtained from cervical cancer cells of Henrietta Lacks, the first immortal cell line, is widely used by the researchers to explore natural safer alternatives and advance the treatment options [5].

MATERIALS AND METHODS:

Selection and Collection of Plant Materials: At first with the help of literature review and published articles, two plants named *Tectona grandis* and *Annona squamosa*, were selected, and then the fully mature fresh leaves were collected from Pabna and Narayanganj districts respectively in the year of 2024.

Preparation of Extracts: The leaves were washed thoroughly, shade dried for 7 days and finely powdered with the help of suitable blender. 50 g dried powder of each plant were macerated for 7 days in ethanol to get crude extract. After filtration with cotton and filter paper, the filtrates were evaporated using rotary evaporator. Then the mass of the extract was determined and the yield was calculated and expressed in percentage.

Yield (%) = Mass of crude extract × 100/ Total mass of dry powder

The mass of *Tectona grandis* crude extract was 2.2 gm and it gave yield of 4.4% and for *Annona squamosa* crude extract was 2.4 gm giving yield of 4.8%. The extracts ware transferred to two closed containers separately for further use and protection.

Phytochemical Analysis:

Qualitative Analysis: The extracts obtained in extraction process were analyzed systematically for the different chemical constituents to assess the active constituents present like carbohydrates, glycosides, saponins, steroids, flavonoids, tannins, phenols, coumarins, resins, and alkaloids [6].

Screening Methods: Some common standard tests that are available for such phytochemical screening are described below.

Test for Carbohydrates: Extracts were dissolved individually in 5ml of distilled water and filtered. The filtrates were used for the following test.

Molisch's Test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution, formation of violet ring at the junction generally indicates the presence of carbohydrates.

Fehling Test: 2ml of each extracts were hydrolyzed with dilute HCl and neutralized with alkali & heated with Fehling's solution A and B, formation of red precipitate indicates the presence of reducing sugars.

Benedict's Test: Filtrates were treated with Benedict's reagent and heated gently, orange red precipitate indicates the presence of reducing sugars.

Test for Glycosides:

General Test: A small amount of an alcoholic extracts were dissolved separately in 1 ml of water and a few drops of aqueous sodium hydroxide solution was added. Development of yellow color was confirms in the presence of glycosides.

Test for Saponins: 20 ml of distilled water was added to 5 ml extract and shake well and wait for 15 min, foam formation indicates positive test.

Test for Steroids: 1ml extract was dissolved in 10 ml of chloroform & equal volume of concentrated H_2SO_4 acid was added from the side of test tube. Result gave two layers, Upper layer shows red whereas lower layer yellow with green fluorescence, indicates positive test.

Test for Flavonoids:
Alkaline Reagent Test: Extracts were treated with 10 % NaOH solution, formation of intense yellow color indicates presence of flavonoids.

Test for Tannins:
Ferric Chloride Test: 4 ml of extracts were reacted with $FeCl_3$ solution gives green color, indicates positive test.

Test for Phenols:
Ferric Chloride Test: Test extracts were treated with 4 drops of Alcoholic $FeCl_3$ solution. Formation of bluish black color indicates the presence of phenols.

Test for Coumarines: 3 ml of 10% NaOH was added to 2 ml of aqueous extracts separately. Formation of yellow color indicates coumarines.

Test for Resins: Using gentle heat, a small quantity of ethanolic extracts were dissolved in 5 to 10 ml of acetic anhydride. After cooling and 0.05 ml of sulphuric acid was added. If resins are present, a bright purplish red color, rapidly changing to violet, is produced for resin[7].

Test for Alkaloids:
General Laboratory Tests: About 0.5 gm extracts were stirred with 5 ml of 1 % hydrochloric acid on a steam bath and filtered. 1ml of filtrates were treated with a few drops of the following reagents separately. Turbidity or formulation of the respective colored precipitates indicates the presents of alkaloids in the extracts.

Mayer's Reagents (Potassium-Mercuric Iodide Solution): White or creamy white precipitate suggests the presence of alkaloids [8].

Qualitative Anticancer Activity:
Used Instruments and Consumables:
Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA), Carbon dioxide Incubator (Nuaire, USA), Trinocular microscope with camera (Optika, Italy) and Hemocytometer. 48-well plate, 96- well plate, 15-ml tubes, Tips, Gloves, Culture flask, Cell culture media, Antibiotics (P+S), Gentamycin, Serological pipette and Trypsin etc.

Sample Preparation: In order to investigate anticancer activity 2% solution was prepared by using distilled water and Dimethyl sulfoxide (DMSO). Then 20mg crude extract of *Tectona grandis and Annona squamosa* were used to prepare solution of 5 mg/ml which were used in qualitative anticancer activity evaluation against HeLa cell.

Maintenance of the Cell Line: Anticancer activity was examined in Centre for Advanced Research in Science using their commercial services. In brief, HeLa line was maintained in DMEM (Dulbecco's Modified Eagles medium) which contained 1% penicillin- streptomycin (1:1) and 0.2% gentamycin and 10% fetal bovine Serum (FBS).

Procedure: Same methods and facilities were employed for *Tectona grandis* and *Annona squamosa*. HeLa Cells ($4.0\times 10^{\wedge 4}$ / 200 µl) were seeded using 48-well plate and incubated at 37°C + 5% CO_2. Next day, 50µl filtered samples were added each well respectively. Cytotoxicity was examined under an inverted light microscope after 48h of incubation. Duplicate wells were used for the sample [5].

RESULTS AND DISCUSSIONS:
Preliminary Phytochemical Screening: Preliminary phytochemical analysis revealed the presence of steroids, tannin and alkaloids in the ethanolic extract of leaves of *Tectona grandis* whereas carbohydrates, glycosides, saponin, flavonoids, phenol, coumarine, resins were not detected.

In contrast, a previous study reported the presence of carbohydrate, glycosides, saponin, steroids, flavanoids, tannin, phenol, coumarine, resins, alkaloids in ethanolic leaves extract of the same plant [7]. These observed variations may be due to the differences in geographical areas, seasonal factors and extraction procedures *etc*.

The result of phytochemical analysis of *Tectona grandis* is represented in the following table:

TABLE 1: RESULT OF PRELIMINARY PHYTOCHEMICAL SCREENING OF *TECTONA GRANDIS*

Sl. no.	Phytochemicals	Observations
1	Carbohydrate	-
2	Glycosides	-
3	Saponin	-
4	Steroids	+
5	Flavanoids	-
6	Tannin	+
7	Phenol	-
8	Coumarine	-
9	Resins	-
10	Alkaloids	+
	+ = Present; - =Absent	

The result of preliminary phytochemical screening of *Annona squamosa* is represented in **Table 2**:

TABLE 2: RESULT OF PRELIMINARY PHYTOCHEMICAL SCREENING OF *ANNONA SQUAMOSA*

Sl. no.	Phytoconstituents	Observations
1	Carbohydrate	+
2	Glycosides	+
3	Steroids	+
4	Tannin	+
5	Alkaloid	+
6	Resin	-
7	Saponin	-
8	Flavonoid	+
9	Phenol	+
10	Coumarin	+
	+ = Present; - =Absent	

The **Table 2** shows the phytochemical constituents present in the ethanolic leaves extract of *Annona squamosa*. It is revealed that carbohydrates, glycosides, steroids, tannins, alkaloids, flavonoids, phenolic compounds, and coumarins were present in the extract. Whereas resins and saponins were absent. The findings of the preliminary screening is supported by another previous experiments [9].

From the result of phytochemical investigation, it is demonstrated that Anno*na squamosa* contained a higher diversity of secondary metabolites compared to *Tectona grandis*.

Qualitative Anticancer Activity: The result of qualitative analysis of anticancer activity of *Tectona grandis* and *Annona squamosa* are initially represented in **Table 3**. It has been observed that fewer of HeLa cells was survived in case of *Tectona grandis* compared to *Annona squamosa*, 48 hours after the administration of those extracts. The finding indicates that *Tectona grandis* possesses superior anticancer activity compared to the other plant.

TABLE 3: RESULT OF QUALITATIVE ANTICANCER ACTIVITY

Sample ID	Survival of HeLa Cells
Solvent -	100%
Solvent +	> 95%
Tectona grandis	< 5%
Annona squamosa	10-20%

Consequently, microscopic images are included here to highlight the cellular changes observed following treatment and to support a more precise interpretation of the results.

A: Solvent (-) **B: Solvent (+)** **C: TG (Before wash)** **D: TG (After wash)** **E: AS**

FIG. 1: EFFECT OF TG (*TECTONA GRANDIS*) AND AS (*ANNONA SQUAMOSA*) ON HELA CELL LINE AFTER 48 HOURS INCUBATION. Here, A: Hela cells control = solvent - (survival rate 100%); B:1 ml Dimethyl sulfoxide (DMSO control = solvent + (survival rate>95%) C: Ethanolic extract of *Tectona grandis* before wash. D: Ethanolic extract of *Tectona grandis* after wash (survival rate <5%); E: Ethanolic extract of *Annona squamosa* (survival rate 10-20%).

Microscopic evaluation demonstrated that in negative and solvent control the normal cellular morphology and cellular density of HeLa cells were maintained with confluency. Survival of cells was 100% as well as 95% respectively in those cases. On the other hand, HeLa cells treated with *Tectona grandis* extract showed noticeable structural alteration, loss of cell adhesion and formation of cellular debris. After washing with phosphate buffered saline to reduce the cellular debris, only a small number of cells remained attached to the culture surface. Cell viability was reduced to ≤5% after 48 hours of treatment which indicates pronounced cytotoxic activity of the extract.

In case of *Annona squamosa* showed some degree of alterations in cellular morphology compared with normal healthy HeLa cells. But, compared with *Tectona grandis*, it showed moderate toxicity. In this case, more round cells and cellular adhesion were observed which indicates only moderate toxicity. So, the cell viability was increased from 10 to 20%.

The anticancer effects observed in this study may be associated with the presence of secondary metabolites that may possess anticancer potential.

CONCLUSION: The current study demonstrated that compared to *Annona squamosa*, *Tectona grandis* is more toxic to HeLa cells. It can be a natural source of potential anticancer agents. It requires further investigation including *in-vivo* assessments followed by isolation and characterization the active compounds responsible for anticancer effects.

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CONFLICT OF INTEREST: The authors declare that they have no conflicts of interest

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