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Pharmacognostical, phytochemical and pharmacological potentials of *Cannabis sativa* L.

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Abstract: *Cannabis sativa* contains promising groups of cannabinoids with reported anticancer properties. For the evaluation of pharmacognostical, phytochemical and pharmacological potentials, the herb was collected from the Department of Narcotics Control, Government of Bangladesh. The plant material was extracted with hexane, ethylacetate, methanol and water separately using sohxlet apparatus. The macroscopic and microscopic examinations of the plant sample followed by phytochemical screenings of different solvent extracts showed the presence of flavonoids, phenols, sterols and terpenoids in major. The extracts were further studied for biological screening in brine shrimp lethality as a mean of anticancer activity using vincristine sulfate as positive standard. The LC_{50} of the hexane part (0.398±0.001 µg/ml) and ethyacetate extract (0.450±0.003 µg/ml) exhibited potential activity in comparison to vincristine sulfate (0.316±0.002 µg/ml). The LC_{50} values of hexane and ethyacetate extracts are indicative for anticancer activity, and the presence of terpinoids like compounds in those extracts are indicative of potential functionality of cannabinoids like compounds.

গাজা বা ভাং(ক্যানাবিস স্যাটাইভা)-র মধ্যে যথেষ্ট পরিমাণ ক্যানাবিনয়েড্ জাতীয় উপাদান থাকায় ক্যান্সার রোগ প্রতিরোধ করার ক্ষমতা রাখে বলে গবেষণায় জানা গেছে। ফার্মাকোগনোস্টিক্যাল, ফাইটোকেমিক্যাল ও ফার্মাকোলজিক্যাল সম্ভাবনাগুলো মূল্যায়ণের জন্য এই ভেষজ্ উপাদানটি বাংলাদেশ সরকারের মাদক নিয়ন্ত্রণ অধিদপ্তর থেকে সংগ্রহ করা হয়েছিল। সেই লক্ষে উপাদানটি হেক্সেন, ইথাইলএসিটেট, মিথানল এবং পানি দ্বারা আলাদাভাবে নিষ্কাশন করা হয়। ম্যাক্রোস্কোপিক ও আনুবীক্ষণিক পরীক্ষাসমূহের পাশাপাশি উক্ত ভেষজ উপাদানের বিভিন্ন দ্রাবক থেকে প্রাপ্ত নির্যাসে ফাইটোকেমিক্যাল পরীক্ষার ফলে ফ্লাভোনয়েড্, ফেনল, স্টেরোল এবং টার্পিনয়েড জাতীয় মূল রাসায়নিক উপাদানের উপস্থিতির প্রমাণ পাওয়া গেছে। প্রাপ্ত নির্যাসগুলো সদ্য ফোটা চিংড়ি মাছের বাচ্চার ওপর পরীক্ষা করা হয়, এটা বোঝার জন্য যে নির্যাসগুলোর ক্যান্সার রোগ প্রতিরোধ করার ক্ষমতা রয়েছে কিনা। এই পরীক্ষার উদ্দেশ্য হলো মাত্রার ওপর নির্ভর করে বাচ্চাগুলো মারা যাবে এবং তার থেকে নির্ণয় করা হবে নির্যাসগুলোর ক্যান্সার রোগ প্রতিরোধ করার ক্ষমতা। আর এখানে নিশ্চিত মানদন্ড হিসাবে ভিংক্রিস্টিন সালফেট ব্যবহার করা হয়েছে। পরীক্ষায় দেখা গেছে হেক্সেন নির্যাস

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০.৩৯৮±০.০০১ মাইক্রোগ্রাম/মিঃলিঃ মাত্রায় এবং ইথাইলএসিটেট নির্যাস ০.৪৫০±০.০০৩ মাইক্রোগ্রাম/মিঃলিঃ মাত্রায় সদ্য ফোটা চিংড়ি মাছের বাচ্চার অর্ধেক সংখ্যায় মেরে ফেলে, যার ফলাফল ভিংক্রিস্টিন সালফেটের (০.৩১৬±০.০০২ মাইক্রোগ্রাম/মিঃলিঃ) সাথে তুলনীয়। এই পরীক্ষালব্ধ ফলাফল এই নির্দেশনা প্রমাণ করে যে, হেক্সেন নির্যাস ও ইথাইলএসিটেট নির্যাস ক্যান্সার রোগ প্রতিরোধ করার ক্ষমতা রাখে। আর ফাইটোকেমিক্যাল পরীক্ষায় এটা প্রমাণ করা হয়েছে যে, নির্যাসগুলোর মধ্যে যে প্রাকৃতিক টার্পিনয়েড রয়েছে তা আসলে ক্যানাবিনয়েড জাতীয় উপাদান যা' প্রকৃতপক্ষে ক্যান্সার রোগ প্রতিরোধ করার ক্ষমতা প্রদর্শন করে।

Keywords: *Cannabis sativa,* pharmacognostical, phytochemical, pharmacological potential, brine shrimp lethality, anticancer activity screening.

INTRODUCTION

Breast cancer, the most common type is resulting with more than 234,000 cases expected in the United States in 2015 (Hernandez et al., 2010). Cancer-related death rates in Bangladesh to be 7.5% in 2005 and 13% in 2030 was estimated by International Agency for Research on Cancer (Hossain & Sullivan, 2013). As compared to other types of cancer, breast cancer has been reported as the highest prevalence rate (19.3 per 100,000) among Bangladeshi women between 15 and 44 years of age (Forazy & Chowdhury, 2015). Upon literature review of the plant as medicinal purpose, it is seen that endo-cannabinoids can act as selective inhibitors of human breast cancer cell proliferation through a growth factor-dependent mechanism (D. Melck et al., 2000; De Petrocellis et al., 1998). It has been reported that cannabinoids can act through different cellular mechanisms by inducing apoptosis, cell-cycle arrest, or cell growth inhibition, targeting angiogenesis and cell migration (Bifulco & Di Marzo, 2002; Guzman, 2003; Kogan, 2005).

The most important source of cannabinoids is the plant *Cannabis sativa* L., an annual herbaceous plant belonging to the family *Cannabaceae,* which has been used as an herbal remedy for centuries. The oldest evidence of cannabis medical use dates back to the ancient China, where it was recommended for rheumatic pain, constipation, disorders of the female reproductive tract, and malaria among other conditions. In traditional Indian Ayurvedic medicine, cannabis was used to treat neurological, respiratory, gastrointestinal, urogenital, and various infectious diseases (Touw, 1981). The plant *Cannabis* has been used as an herbal remedy for centuries and is the most important source of phytocannabinoids. The endocannabinoid system (ECS) consists of receptors, endogenous ligands (endocannabinoids) and metabolizing enzymes, plays an important role in different physiological and pathological processes (Dariš et al., 2019) such as control of cardiovascular tone, energy metabolism, immunity, and reproduction (Pacher et al., 2006; Pertwee, 2009). Cannabinoids are a large and important class of complex compounds that have an encouraging therapeutic potential for the treatment of diverse of diseases, including breast cancer (Baroi et al., 2020). In this study, focuses has been given on evaluation of pharmacognostical, phytochemical and pharmacological potentials of *Cannabis sativa* L that provide an evidence for anticancer effects of plant-derived cannabinoids.

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MATERIAL AND METHODS

Collection of plant material: About 260gm of the plant material *Cannabis sativa* was collected from the Department of Narcotics Control, Government of Bangladesh with prior permission from the authority. According to their information, the sample was collected from Kustia, Bangladesh. Identification of the herbs was done at the Bangladesh National Herbarium, Dhaka vide accession No. DACB 38696 and an authentic herbarium specimen was deposited in the herbarium museum for future reference.

Pharmacognostic study: Authenticated plant sample was taken into consideration for pharmacognostical analysis, such as macroscopic and microscopic studies. It was then grinded into powder and sieved to yield of different size grades using mesh # 10, 20, 30 and 40 (size distribution shown in Table 1). Coarse (#10 mesh) and fine (#40 mesh) powders were used to find different characteristics such as smell, taste, appearances, cell and vessels as per standard procedures (Kokate, 2005; Bhatia et al., 2008).

Solvent extraction: The powdered sample was weighed and packed for extraction. Percolation technique was used for extraction using soxhlet apparatus (Harwood & Moody, 1989). Four different solvents of different polarities viz. hexane, ethylacetate, methanol and water were used and 10 cycles were run for each of the solvent. The non-soluble portion of the extracted solid remaining in the thimble was dried and returned to the Department of Narcotics Control as per direction. The solvent of extracts was evaporated using rotary evaporator. The extracts were further freeze-dried to remove moisture, which provides stability of the compounds. It was then weighed (Table 2) and stored in a cool dark place for further use.

Table 1: Size distribution of powdered sample of *Cannabis sativa* L after sieving

Mesh Number	Amount of powder (gm) (%)
40	231.01 (92.40)
30	10.71 (4.28)
20	4.66 (1.86)
10	3.39 (1.36)

Table 2: Percent of extract yielded after rotary evaporation and freeze drying after extraction with hexane, ethyl acetate, methanol and water

Name of extract	Amount (gm) (%Yield)
Hexane extract	20.59 (8.24)
Ethyl acetate extract	05.90 (2.36)
Methanol extract	06.50 (2.60)
Water extract	7.90 (3.16)

Phytochemical screening of the samples: Different solvent extracts of the herb were then subjected to different qualitative tests to ascertain the presence of various phytoconstituents (Evans, 2009; Harborne, 1984; Ugochukwu et al., 2013).). At first a few milligram of sample extracts was dissolved in suitable solvents. It was then filtered to remove any gritty particles or other insoluble portion and

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then three sets of experiments were performed on the filtrate. The processes employed are described below:

Test for Alkaloids: The residue of each extract was taken separately in 5 ml of 1.5 % w/v hydrochloric acid and filtered, which were then used for following alkaloid detection using following tests- Mayer's Reagent test (Evans,1997), Wagner's Reagent test (Wagner, 1993) and Hager's Reagent test (Wagner et al., 1996).

Test for Carbohydrates and glycosides has been performed with Molisch's test, Benedict's test, Barfoed's test, Borntrager's test and Legal test (Evans,1997), Keller Kelliani's test (Ramkrishnan & Rajan, 1994).

Test for flavonoids has been done with Alkaline reagent test (Trease & Evans, 2002; Kokate, 2014), Shinoda test (Trease & Evans, 2002).

Phenols were detected following Ferric chloride test (Trease & Evans, 2002).

Test for tannins has been performed with precipitate test (Kokate, 2004).

Test for proteins and amino acids (Fisher, 1968) were confirmed using Ninhydrin test (Yasuma & Ichikawa, 1953), Biuret test (Gahan, 1984) and Millon's reagent test (Ruthmann, 1970).

The saponins were detected by emulsification test (Segelman & Farnsworth, 1969).

Test for sterols was performed with Liebermann-Burchard test (Sofowora, 1993; Finar, 1986).

The tannins were detected with Braymer's test (Segelman & Farnsworth, 1969).

Test for terpinoids has been performed using Salkowki's test (Trease & Evans, 2002).

The presence of quinones was determined following precipitation (or coloration) test (Kokate, 2004; Khandelwal, 2000).

Test for oxalates has been performed coloration test (Kokate, 2004; Khandelwal, 2000).
Brine-shrimp lethality bioassay test-

Brine shrimp lethality bioassay (Meyer, 1982): has been performed for predicting cytotoxicity (Ghisalberti, 1993). Brine shrimp (*Artemia salina Linn.*) eggs were hatched in artificial seawater, which was prepared by dissolving 38g of sea salt in 1.0 L of distilled water. The pH of the solution was adjusted to 8.5. After 24hr of incubation period at room temperature (26-30° C) under constant aeration, the larvae (nauplii) came out. They were attracted to one side of the vessel with a light source and collected with a pipette for assay. The bioactivity of the extracts was monitored by the brine shrimp lethality. Eleven washed and numbered test tubes were taken for each sample. Each fraction was dissolved in dimethylsulfoxide (DMSO) to prepare a concentration of 40 µg/ml in first tube, 20 µg/ml in second tube, 10 µg/ml in third tube and so on. The concentration was in the range of 40 – 0.078 µg/ml. Then ten larvae were counted and put into each of them and the final volume was adjusted to 10 mL by adding artificial sea saltwater. Then the test tubes were incubated for 24h. The tubes were then examined under a magnifying glass and the number of dead nauplii in each tube

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counted. Vincristine sulfate was used as a positive control in all experiments. Measured amount of the Vincristine Sulfate (2mg)was dissolved in DMSO to get an initial concentration of 20 µg/ ml from which serial dilutions are made using DMSO to get 10 µg/ ml, 5 µg/ ml, 2.5 µg/ ml, 1.25 µg/ ml, 0.625 µg/ ml, 0.3125 µg/ ml, 0.15625 µg/ ml, 0.078125 µg/ ml, 0.039 µg/ ml. Experiments were conducted for 3 times and the average values were used to determine the LC_{50} value for each of the extracts.

Gel permeation chromatography (van der Jagt, 2013; Striegel, 2013): The bioactivity study revealed the hexane extract might have promising functionality and was taken into consideration for further separation. Gel permeation chromatography was used to fractionate them using Sephadex (LH20) as stationary phase. The stationary bed was prepared with activated Sephadex and a solvent system of 2:5:1 mixture of hexane, dichlromethane and methanol was used to dissolve the sample and subsequently applied to the column with the help of pasture pipette. The column was then eluted with the same solvent mixture and then gradually changed towards a more polar solvent system ending with 100% methanol. Fractions were collected in properly washed test tubes filling around 2.5mL.
Analysis of column fractions by TLC-
All the column fractions were screened by TLC under UV light followed by spraying with vanillin-sulfuric acid reagent. A number of compounds were detected. The compounds present in the fractions with similar R_f values in the chromatograms were added up and repeated the procedure.

Analysis of column fractions by HPLC: The added up sample fractions were dissolved in acetonitrile. Separation, detection and analysis have been performed in high performance liquid chromatography equipped with a manual injector, a vacuum degasser, a multiple-wavelengths UV/Visible detector (Shimadzu SPD 20A, Japan) and an ODS column Capcell Pak (150 mm×4.6 mm I.D., 5 µm particle size) were used for chromatographic analyses). Then it was transferred to 1.5 ml HPLC vials. Then high performance liquid chromatography was performed on them. The column condition was: injection volume 30µl, flow rate 1 ml/min, UV 210nm, solvent system with 90% acetonitrile and 10% water isocratic for 5 min, 90% aqueous acetonitrile to 100% acetonitrile for 10 min and 100% acetonitrile for 10 min. Total run time was 25 minutes.

Statistics: The results of the brine shrimp lethality assay were stated as the mean value ± SD (n = 3). The one-way ANOVA test was done to determine the significant mean difference (n = 3) between the samples followed by Tukey's multiple comparison test using graph pad prism 6.0.

RESULTS AND DISCUSSION

Pharmacognostical analysis of *Cannabis sativa* L showed that the coarse powder (#10 mesh size) of the plant material exhibited with characteristic smell and taste. In the microscopic view of the plant confirmed the presence of elongated and glandular hairy appearance. The fine powder (# 40 mesh size) was mounted in glycerin with staining agent and observed under microscope, showed presence of different types of cells, vessels and others.

The hexane, ethylacetate, methanol and water extracts of *Cannabis sativa* L were subjected to preliminary phytochemical screening following standard methods for determination of alkaloid, carbohydrate, sterols, proteins, flavonoids, glycosides, tannins, terpenoids, saponins, oxalates, quinones (Table 3), out of which mainly revealed the presence of flavonoids, phenols, sterols and terpenoids were in major.

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In the brine shrimp lethality bioassay study on the hexane, ethyl acetate, methanolic extracts of *Cannabis sativa* showed positive results, indicating that the test samples are biologically active and the hexane extract showed the best profile. On the other hand, the water extract doesn't have any bioactivity. Each of the test samples showed different mortality rates at different concentrations. Percent mortality is directly proportional to concentration (in logarithmic scale) of test samples. From the graphs (Figure 1), the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the test samples. Among the extracts, the hexane extract exhibited LC_{50} of 0.398±0.001 µg/ml**,** ethyacetate extract exhibited 0.450±0.003 µg/ml and methanol extract showed 19.95±1.542 µg/ml which are comparable to positive control vincristine sulfate (0.316±0.002µg/m) (Table 4).

Table 3: Result of phytochemical screening of hexane, ethylacetate, methanol and water extracts

Tests	Name of test	Hexane extract	Ethylacetate extract	Methanol extract	Water extract
Test for Alkaloids	Wagner's reagent	-	-	++	+
	Mayer's Test	-	-	++	+
	Hager's Test	-	-	++	+
Test for Carbohydrates	Molisch's test	-	-	+	++
	Benedict's Test	-	-	-	+
	Barfoed's Test	-	-	+	++
Test for glycosides	Keller Kelliani's test	-	-	-	-
	Legal's Test	-	-	-	-
	Borntrager's Test	-	-	-	-
Test for Flavonoids	Alkaline reagent test	+++	+++	+++	++
	Shinoda's Test	++	+++	+++	-
Test for Phenols	Ferric chloride test	+++	+++	+++	++
Test for tannins	Precipitate test	-	-	-	++
Test for Amino acids and Proteins	Ninhydrin test	-	-	+	-
	Biuret Test	-	-	-	+
	Millon's Test	-	-	-	+
Test for Saponins	Emulsification test	-	-	++	++

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Test for Sterols	Liebermann-Burchard test	+++	++	-	-
Test for Tannins	Braymer's test	-	-	+	++
Test for Terpenoids	Salkowki's test	+++	+++	+++	+
Test for Quinones	Precipitate test	-	-	+	+
Test for Oxalate	Coloration test	-	-	-	-

The LC_{50} values of hexane and ethyacetate extracts are indicative of their anticancer activity which is almost similar to vincristine sulfate may be due to the presence of terpinoid like compounds in those extracts, which are related of the compounds like cannabinoids as anticancer agents (Velasco et al., 2016). As the hexane extract exhibited maximum activity, it was subjected for Gel permeation chromatographic separation technique. All the column fractions were screened by TLC under UV light followed by spraying reagent. The fractions presenting similar chromatograms were mixed up and chromatographed again. Finally the mixed up factions were detected using HPLC technique. From the chromatograms a good separation of the cannabinoids has been observed with their following approximate retention times (Rt in min), such as cannabidiolic acid (CBDA, Rt 7.23 min), cannabigerol (CBG, Rt 8.32 min), cannabidiol (CBD, Rt 9.95 min), *delta*-9-tetrahydrocannabivarin (THCV, Rt 11.37 min), *delta*-9-tetrahydrocannabinol, (D9-THC, Rt 16.19 min), cannabicyclol (CBL, Rt 18.93 min) and cannabichromene (CBC, Rt 19.93 min). Though the representative chromatogram of the standard cannabinoids was not done, but separation of the compounds is almost similar to findings of Gul et al. (2015) following their retention times (Backer et al., 2009).

Table 4: LC_{50} value of the hexane extract, ethyl acetate extract, methanol extract, water extract and standard Vincristine sulfate

Test samples	**Regression line**	**R^2**	**LC50 (µg/ml) (Mean ± SD)**
Water extract	Nil	Nil	Nil
Hexane extract	y = 4.5389x + 7.164	R^2 = 0.987	0.398±0.001
Ethyl acetate extract	y = 36.003x + 63.016	R^2 = 0.976	0.450±0.003
Methanol extract	y = 40.342x - 2.112	R^2 = 0.989	19.95±1.542
Vincristine sulfate	y= 37.89x + 68.695	R^2 = 0.988	0.316±0.002

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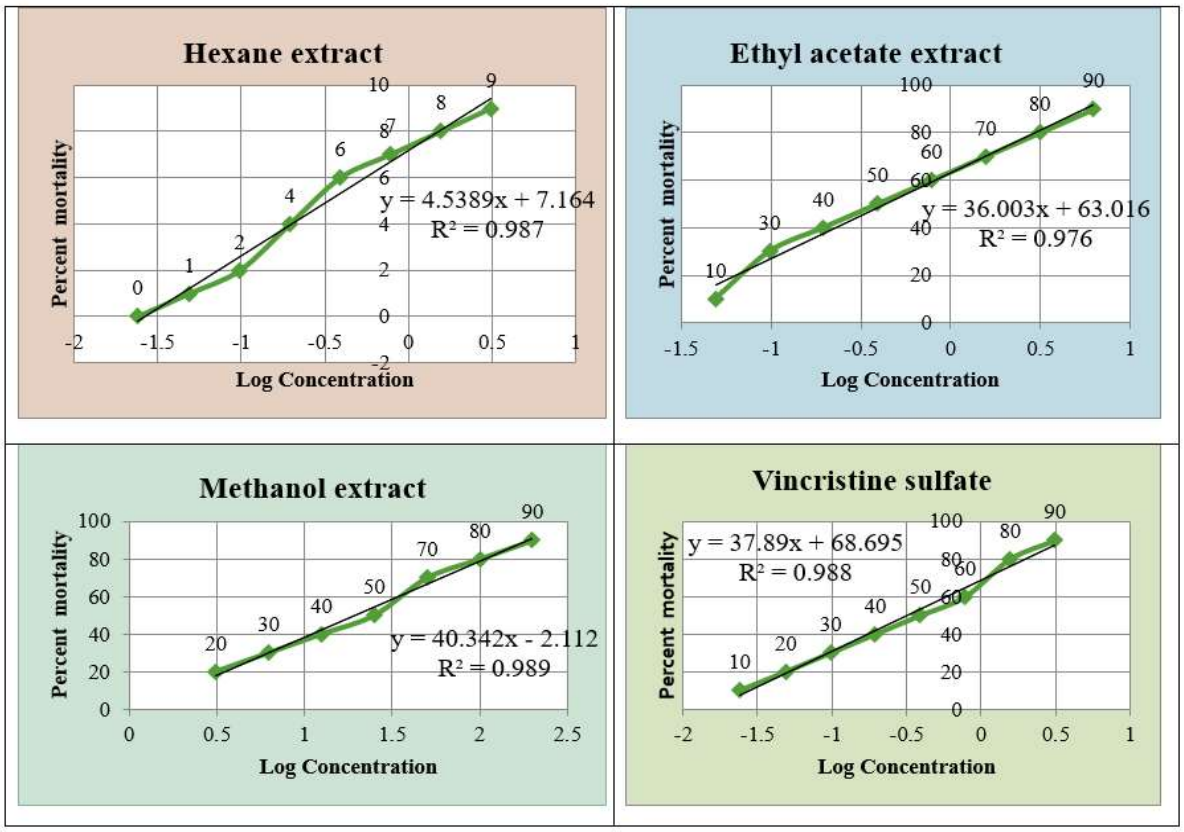

Figure 1: Percent mortality vs log of concentration plot for determination of LC_{50} value of hexane extract, ethyl acetate extract, methanol extract and Vincristine sulfate

CONCLUSION

The pharmacognostical and phytochemical screening of *Cannabis sativa* with its hexane, ethylacetate, methanol and water extracts revealed the importance of terpenoids (might be cannabinoid compounds) for exhibiting promising lethality (shrimp lethality bioassay) which is comparable to standard vincristine sulfate.

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DECLARATION OF CONFLICT OF INTEREST

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The authors declared that there have no conflict of interest among them

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