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Comparative Pharmacological Evaluation of Mangrove Plant *Xylocarpus mekongensis* Pierre and Associated Fungus

Sadia Airin, Rahul Dev Bairagi, Sharika Noshin, Md. Sohanur Rahaman, Ritu Porna Biswas, Amit Kumar Acharzo, and Md. Amirul Islam

ABSTRACT

***Xylocarpus mekongensis* commonly known as Poshur, is an evergreen mangrove plant originating from Asia, Indonesia, the Southwestern Pacific Islands, and northern Australia. This study was aimed at compiling information on the comparative pharmacological properties of the methanolic bark extract of *X. mekongensis* and the fungal endophyte based on their antioxidant, antimicrobial, and cytotoxic activity. The plant extract showed more phenolic (277 mg GAE/g) and flavonoid (140 mg QE/g) content than the fungal extract (45 mg GAE/g and 76 mg QE/g, respectively). The bark extract exhibited better DPPH scavenging capacity (IC_{50} = 28.27 µg/ml) than endophyte XMSF-I (IC_{50} = 143.46 µg/ml) extract. Furthermore, we observed that the endophytes associated with this plant showed more significant antimicrobial activity against *E. coli, S. aureus, B. subtilis*, and *A. brasiliensis* than its bark extract. In the brine shrimp lethality bioassay, the bark extract and endophyte revealed diminutive lethality (214 and 286 µg/ml, respectively) in comparison with standard vincristine sulfate (0.44 µg/ml). Hence, methanolic bark extract showed more positive reviews than associated fungi.**

Keywords: Antioxidant, Antimicrobial, Cytotoxic, Fungus, Sundarban, *Xylocarpus mekongensis.*

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I. Introduction

Mangrove plants exhibit biochemical distinctiveness, characterized by the synthesis of a diverse range of natural compounds that possess exceptional bioactivity [1]. The compounds they contain possess active metabolites characterized by unique chemical structures that span a wide range of chemical classes, including alkaloids, phenols, steroids, terpenoids, tannins, and others [1]. *Xylocarpus mekongensis*, or puzzle fruit tree, or poshur, is a plant of mangrove areas; it is prominently found in the littoral forests of Bengal, Burma, Andaman, Malay, Singapore, and Cambodia [2]. This plant enjoys vast folklore uses as traditional medicine. The bark is used as an antimalarial, antidiarrheal, antinociceptive, anti-inflammatory, and antioxidant [3]. The fruit is used to treat elephantiasis and prevent swelling of the breast [4].

Endophytic fungi are microorganisms present in the living tissues of diverse plants, establishing a mutually beneficial symbiotic relationship [5]. Endophytes have been identified as abundant reservoirs of bioactive metabolites [6]. The secondary metabolites produced by microorganisms in general and endophytic microorganisms in particular have been studied and explored for various industrial applications,

including pharmacological and clinical uses [7].

Microorganisms from various taxonomic groups, including fungi, bacteria, and actinomycetes, have been commonly observed in endophytic symbiosis with plants. They enhance the synthesis of secondary metabolites through various biological activities. Fungi are capable of producing a wide range of unique secondary metabolites, many of which possess significant biological activities that can be utilized for human health and well-being. Certain endophytic microorganisms have the ability to produce secondary metabolites that are similar to those produced by the host plant. This characteristic makes them a potentially valuable source of new compounds [8].

The Sundarban, the world's biggest mangrove forest, is on the verge of extinction as a result of climate change, deforestation, soil erosion, and damaging natural disasters [9]. Therefore, the determination of the antioxidative, antimicrobial, and cytotoxic activity of both *X. mekongensis* and XMSF-1 [10] extract is significant and is commencing for new drug development.

II. Materials and Methods

A. Chemicals

Reagents of an analytical grade, such as 70% ethanol, gallic acid, folin-ciocalteu (FC) reagent, and gallic acid, were used. 7% sodium carbonate, 5% weight-per-volume sodium nitrate, 10% weight-per-volume aluminium chloride, 2,2-diphenyl-1-picryl hydrazyl (DPPH), methanol, 1M NaOH, quercetin, chloramphenicol, dimethyl sulfoxide, ascorbic acid, agar made from potato dextrose, pure sodium chloride, brine shrimp eggs, kanamycin at a concentration of 30 micrograms per disc, fluconazole at a concentration of 50 micrograms per disc, dichloromethane etc.

B. Preparation of Plant Extract

The filtrate obtained (methanol extract) was evaporated. Then, concentrated extract was placed in the beaker, and an opening in the beaker was covered with a perforated sheet of aluminum foil for the evaporation of methanol. The beaker was stored in a dry, cold environment for several days before being evaporated under a table fan, and it was a blackish-green substance. The concentrates were designated as crude methanolic bark extracts of *X. mekongensis*. Cold extraction was carried out on 200 g of bark powder with 96% methanol. The total weight of the dried extract was 12.5 g, and the yield was 6.25%.

C. Isolation and Extraction of Associated Fungus

The endophytic fungi, which were isolated from mangrove plants, were incubated in a broth medium of potato dextrose at 28 °C for 21 days that was shaken at 150 rpm. In the separation process, cotton is used to aseptically separate the micelle from the broth. The broth was transferred to a separatory funnel and added to n-hexane at a 2:1 ratio. Then the diffracted broth was separated from the upper layer with fat. After that, a medium polar solvent called DCM was used to isolate the secondary metabolites that had been extracted from the diffracted broth. Then the extracted liquid evaporated using a rotary evaporator at 37 °C [11].

D. Quantitative Antioxidant Assay

DPPH radical scavenging assay of methanolic bark extracts of *X. mekongensis* and XMSF-1 were carried out using a Thermoscientific Multiskan microplate photometer to measure the absorbance at 517 nm. Determination of antioxidant capacity in terms of IC_{50} value from the curve of log concentration vs. % inhibition [12], [13].

$$\% \text{ inhibition} = [(\text{Blank absorbance} - \text{Sample absorbance}) / \text{Blank absorbance}] \times 100$$

TPC was measured using FC reagent and gallic acid as a standard, and the results were given as mg gallic acid equivalent (mgGAE)/g dried extract of *X. mekongensis* and XMSF-1, respectively [14]. An assay named aluminum chloride colorimetric was used for the quantification of TFC in dried extracts of *X. mekongensis* and XMSF-1, respectively, and quercetin was used as a standard [13], [15].

E. Antimicrobial activity by Disc Diffusion Method

The disc diffusion method was used to test the antibacterial and antifungal activity of *X. mekongensis* and XMSF-1 against two Gram (+ve) *S. aureus*, B. subtilis, and two Gram (-ve) *E. coli*, *S. enterica* bacterial strains, as well as two fungal strains, *C. albicans* and *A. brasiliensis*. All equipment was sterilized by autoclaving at 121 °C and 15 lbs./inch2 pressure for 15 minutes. 100 µL ethanol was applied to the blank disc as a negative control. Three types of discs were used: sample, standard, and blank discs. Each broth plate was divided into four portions, i.e., two for samples (250 µg/100 µL and 500 µg/100 µL); one for standard; and another for blank. Kanamycin (30 µg/disc) and fluconazole (50µg/disc) were used as standard. The plates were incubated at 37 °C for 16–18 hours for antibacterial activity and 48–72 hours for antifungal activity. After proper incubation, the diameter of the zone of inhibition was measured with a calibrated scale [16], [17].

F. Cytotoxic Activity by Brine Shrimp Bioassay

Brine shrimp (Artemia salina) nauplii were used as test organisms in the brine shrimp lethality bioassay to determine the cytotoxic activity of *X. mekongensis* and associated fungi. 38 g of sea salt was dissolved in distilled water to make 1 L and then filtered off. 0.5 g of A. salina eggs were soaked in 1 L of seawater. The airpipe and electric bulb were adjusted for hatching and kept for 18–24 hours. 2 mg of *X. mekongensis* extract was adjusted to 3.125 ml by sea water. DMSO was added, and the concentration became 640 µg/ml (stock solution). The XMSF-1 stock solution was prepared in the same way. 0.25 mg vincristine sulfate (std.) with DMSO and sea water was adjusted to 25 ml, and the concentration became 10 µg/ml. Among 28 test tubes, 14 were taken for samples in the following concentrations (5, 10, 20, 40, 80, 160, and 320 µg/ml), 4 for control, and 10 for standard in different concentrations (5, 2.5, 1.25, 0.625, and 0.3125 µg/ml). However, in each test tube, 10 alive brine shrimp were taken and observed after 24 hours [18], [19].

G. Statistical Analysis

GraphPad Prism 8.0 was used to conduct the antioxidant experiment's statistical analysis. The data were calculated using the mean and SEM of each test parameter. IC_{50} and

LC_{50} were calculated from the equation y=mx+c. For statistical significance, all differences were considered at p=0.05.

III. Results

A. In-Vitro Antioxidant Activity

1) DPPH Free Radical Scavenging Assay

Tested plant extracts and endophytes DPPH free radical scavenging properties using dose-dependent scavenging. The methanolic bark extracts of *X. mekongensis* showed very good inhibition with an IC_{50} value of 28.27 µg/mL in comparison with ascorbic acid (IC_{50} ~14.21 µg/mL) which is a well-known standard. Whereas the endophyte XMSF-1 showed significant inhibition with an IC_{50} value of 143.46 µg/mL.

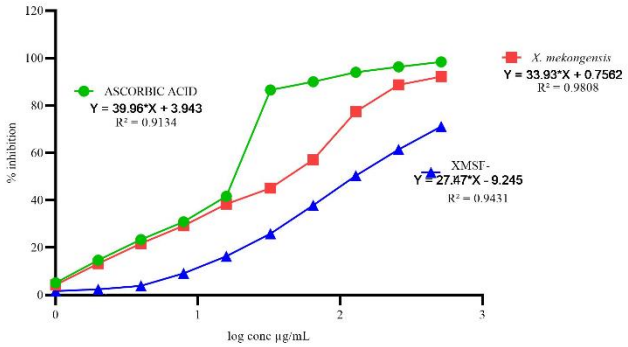

Fig. 1. DPPH scavenging activity of XMSF-1 and *X. mekongensis*.

2) Quantification of Total Phenolic Content (TPC)

Total phenolic content was ~277.34 mg GAE/g in *X. mekongensis* bark extract, which supports that the plant has some biological effects, including antioxidant activity, and total phenolic content was ~44.63 mg GAE/g in XMSF-1, which is much less than the plant.

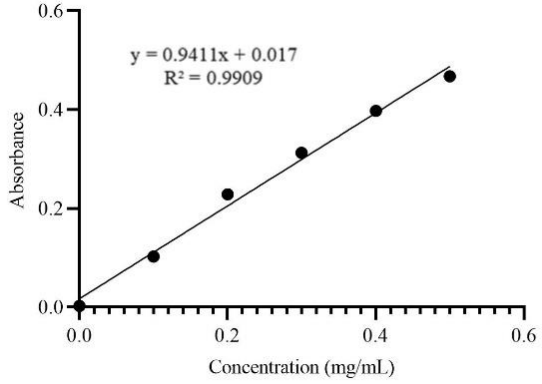

Fig. 2. TPC of *X. mekongensis* & XMSF-1 based on gallic acid standard calibration curve.

3) Quantification of Total Flavonoid Content (TFC)

Total flavonoid content was ~140.17 mg QE/g in *X. mekongensis* bark extract and ~76.01 mg QE/g in XMSF-1, respectively.

B. Antimicrobial Activity

X. mekongensis showed antibacterial activity against *S. aureus* and *B. subtilis* and antifungal activity against *A. brasiliensis*. On the other hand, the endophyte XMSF-1 showed activity against *S. aureus*, *B. subtilis* and *E. coli* and antifungal activity against *A. brasiliensis*.

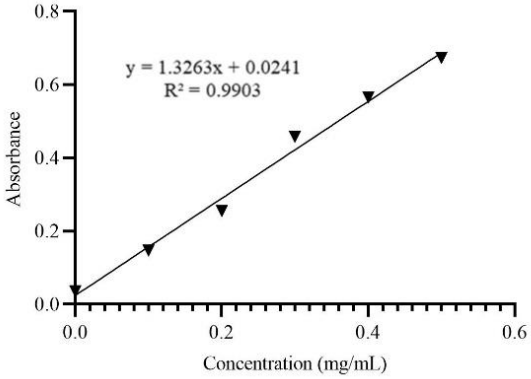

Fig. 3. TFC of *X. mekongensis* & XMSF-1 based on of quercetin standard calibration curve.

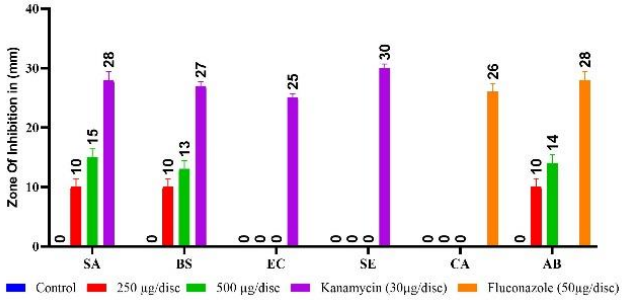

Fig. 4. In vitro antimicrobial activity of *X. mekongensis* extracts by disc diffusion method. (SA: *Staphylococcus aureus*; BS: *B subtilis*; EC: *E. coli*; SE: *S. enterica*; CA: *C. albicans*; AB: *A. brasiliensis*).

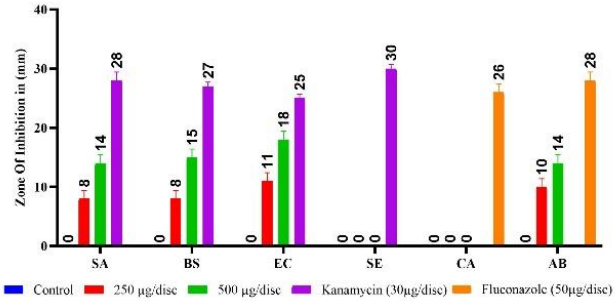

Fig. 5. In vitro antimicrobial activity of XMSF-1 extracts by disc diffusion method. (SA: *Staphylococcus aureus*; BS: *B subtilis*; EC: *E. coli*; SE: *S. enterica*; CA: *C. albicans*; AB: *A. brasiliensis*).

C. Screening for Cytotoxic Activity

In the brine shrimp lethality bioassay, the LC_{50} values of the methanolic bark extract of *X. mekongensis* and endophyte XMSF-1 was 213.51 and 286.50 µg/mL respectively, whereas the LC_{50} value for the standard drug vincristine sulfate was 0.44 µg/mL. Regression analysis showed that the extracts' LC_{50} was significantly lower than vincristine sulfate. While it is still high for a crude extract, this suggests that the extract may contain one or more substances with biological activity.

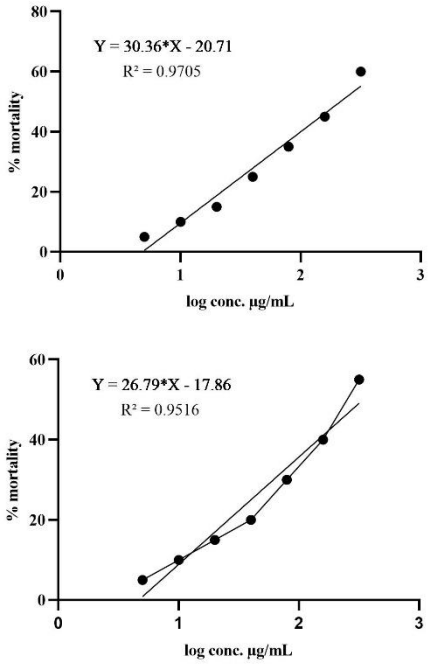

Fig. 6. Cytotoxicity of *X. mekongensis* and XMSF-1.

IV. Discussion

Antioxidant activity is a complex property of the phytoconstituents in a plant extract. In the present study, we have estimated free radical scavenging activity, total phenolic contents, and total flavonoid contents. The quantitative antioxidant assay is based on the ability of a stable free radical, DPPH, which is decolorized in the presence of antioxidants. The change in absorbance of DPPH, an antioxidant compound, allows for the quantitative measurement of its decolorization [20]. The plant extracts (*X. mekongensis*) are able to reduce the stable DPPH free radical in comparison with the standard. According to the findings, phytoconstituents present in plant extracts have a significant impact on donating hydrogen to free radicals in order to neutralize their damaging effects. On the other hand, the endophyte shows little activity in comparison to the standard.

Plant secondary metabolites, polyphenols, are critical indices for determining antioxidant capacity. Phenolics are a type of secondary metabolite, and these phytoconstituents are usually present in plants as proteins such as phenylalanine and tyrosine. Moreover, its hydroxyl groups exert scavenging activity [21]. From the total phenolic content determination assay we found a very good amount of total phenolic content in both the plant and endophyte (277.34 and 44.63 mg GAE/g respectively), which supports the idea that plant has a good antioxidant property. Solvent dependent TPC extracts are expressed as mg GAE/g.

The antioxidant activity of compounds is correlated with flavonoid structure, and as a result, many in vitro and in vivo investigations have been carried out using natural flavonoids. In *X. mekongensis* bark extract and XMSF-1, the total flavonoid content was 140.17mg QE/g and 76.01mg QE/g, respectively.

To find effective pharmacologic activity against bacteria and fungi, the study was carried out. The endophytic XMSF-1 showed good antimicrobial activity compared to the plant extract. In the XMSF-1 extract, activity was observed against *E. coli*, *B. subtilis*, and *S. aureus* whereas the plant extract showed activity against *S. aureus* and *B. subtilis* only. While both the plant and endophyte extracts showed antifungal activity against *A. brasiliensis*. Perhaps concentration played a role in the observed activity in further experiments. To exert antibacterial activity in disc diffusion assays, non-polar compound(s) may also be a reason, as they may fail to diffuse in agar media. According to their study the plant extracts exhibited considerable minimum inhibitory concentrations (MIC) [16]. The result provides support for the claim that both the plant and endophyte have antibacterial and antifungal activity against some bacteria and fungi.

Pharmacological effects of bioactive compounds are of interest now due to the treatment and prevention of cancer diseases. It is generally believed that natural products are good for physiological health. Various flavonoids and nonflavonoids have been reported as showing anticancer, antitumor, and antioxidant activities [22]. Almost 60% of anticancer drugs are isolated from natural sources like plants (i.e., vincristine, irinotecan, and camptothecines) and microorganisms (i.e., doxorubicin, dactinomicines, mitomycin, and bleomycin) [23].

To evaluate cytotoxicity, the brine shrimp lethality bioassay is a quick, easy, and straightforward method for determining the biologically active substances that are contained in a crude extract. It just needs a tiny amount of test material, which is affordable and does not require aseptic methods. This bioassay evaluates cytotoxicity, antibacterial activities, pesticidal effects, and various pharmacologic actions [24]. The findings of this study suggest that chemicals with biological activity, such as those that inhibit enzymes and interfere with ion channels, are found in the bark extracts of *X. mekongensis* and endophytes. These compounds may also have antibacterial, pesticidal, and/or cytotoxic properties [16]. The percentage mortality of the shrimp nauplii increased gradually with concentration for both the test extracts and vincristine sulfate.

V. Conclusions

Based on the current study, the methanolic bark extract of *X. mekongensis* has more phenolic compounds and flavonoids than XMSF-1 and more antioxidative free radical scavenging activity than XMSF-1. On the other hand, compared to the bark extract, XMSF-1 has shown very effective antimicrobial activity. *X. mekongensis* exhibited higher cytotoxicity than XMSF-1 in the brine shrimp lethality test. Thus, fungi from mangrove plants may be a promising approach for bioactive compound identification and new drug development. The results suggest further investigations to isolate and determine the extent of bioactive compounds responsible for the exhibited biological activities.

Abbrebiations

X. *mekongensis*: *Xylocarpus mekongensis*
DPPH: 2, 2-diphenyl- 1-picryldydrazyl
IC_{50}: 50% Inhibitory Concentration
DMSO: Dimethylsulfoxide

GAE: Gallic Acid Equivalent
XFSF-1: *X. mekongensis* Separated Fungus-1
SEM: Standard error of the mean
LC_{50}: median lethal concentration
DCM: dichloromethane

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CONFLICT OF INTEREST

The authors assert that they have no conflicts of interest.

REFERENCES

[1] Patra JK, Thatoi HN. Metabolic diversity and bioactivity screening of mangrove plants: a review. *Acta Physiologiae Plantarum*. 2011 Jul;33:1051-61.
[2] Azad S, Mollick AS, Setu FA, Khan NI. Stand structure, tree species diversity, and leaf morphological plasticity in Xylocarpus mekongensis Pierre among salinity zones in the Sundarbans, Bangladesh. *Journal of Asia-Pacific Biodiversity*. 2022 Sep 1;15(3):414-24.
[3] Bahadur B, Reddy KJ, Rao ML. Medicinal plants: an overview. Adv Med plants Univ Press Hyderabad. 2007.
[4] Das SK, Samantaray D, Thatoi H. Ethnomedicinal, antimicrobial and antidiarrhoeal studies on the mangrove plants of the genus Xylocarpus: a mini review. *J. Bioanal. Biomed*. 2014;12(004).
[5] Wilson D. Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos*. 1995 Jun 1:274-6.
[6] Tan RX, Zou WX. Endophytes: a rich source of functional metabolites. *Natural product reports*. 2001;18(4):448-59.
[7] Conrado R, Gomes TC, Roque GS, De Souza AO. Overview of bioactive fungal secondary metabolites: Cytotoxic and antimicrobial compounds. *Antibiotics*. 2022 Nov 11;11(11):1604.
[8] Ahmed M, Hussain M, Dhar MK, Kaul S. Isolation of microbial endophytes from some ethnomedicinal plants of Jammu and Kashmir. *J Nat Prod Plant Resour*. 2012;2(2):215-20.
[9] Aziz A, Paul AR. Bangladesh Sundarbans: present status of the environment and biota. *Diversity*. 2015 Jul 10;7(3):242-69.
[10] Rahaman MS, Siraj MA, Sultana S, Seidel V, Islam MA. Molecular phylogenetics and biological potential of fungal endophytes from plants of the Sundarbans mangrove. *Frontiers in microbiology*. 2020 Nov 13;11:570855.
[11] Garcia A, Rhoden SA, Bernardi-Wenzel J, Orlandelli RC, Azevedo JL, Pamphile JA. Antimicrobial activity of crude extracts of endophytic fungi isolated from medicinal plant Sapindus saponaria L. *Journal of applied pharmaceutical science*. 2012 Oct 28;2(10):035-40.
[12] Sadhu SK, Okuyama E, Fujimoto H, Ishibashi M. Separation of Leucas aspera, a medicinal plant of Bangladesh, guided by prostaglandin inhibitory and antioxidant activities. *Chemical and pharmaceutical bulletin*. 2003;51(5):595-8.
[13] Nahar MN, Acharzo AK, Rahaman MS, Zabeen IA, Haque S, Islam MA. Phytochemical screening and antioxidant, analgesic, and anthelmintic effect of ethanolic extract of Merremia umbellate stems. *Clinical Phytoscience*. 2020 Dec;6(1):1-1.
[14] McDonald S, Prenzler PD, Antolovich M, Robards K. Phenolic content and antioxidant activity of olive extracts. *Food chemistry*. 2001 Apr 1;73(1):73-84.
[15] Ribarova F, Atanassova M, Marinova D, Ribarova F, Atanassova M. Total phenolics and flavonoids in Bulgarian fruits and vegetables. *JU Chem. Metal*. 2005;40(3):255-60.
[16] Ahmed A, Labu Z, Dey S, Hira A, Howlader M, Hossain M, Roy J. Phytochemical screening, antibacterial and cytotoxic activity of different fractions of Xylocarpus mekongensis Bark. *Ibnosina Journal of Medicine and Biomedical Sciences*. 2013 Aug;5(04):206-13.
[17] Amenu D. Antimicrobial activity of medicinal plant extracts and their synergistic effect on some selected pathogens. *American Journal of Ethnomedicine*. 2014;1(1):18-29.
[18] Vanhaecke P, Persoone G, Claus C, Sorgeloos P. Proposal for a short-term toxicity test with Artemia nauplii. *Ecotoxicology and environmental safety*. 1981 Sep 1;5(3):382-7.
[19] Sleet RB, Brendel K. Improved methods for harvesting and counting synchronous populations of Artemia nauplii for use in developmental toxicology. *Ecotoxicology and environmental safety*. 1983 Oct 1;7(5):435-46.
[20] Korbut O, Bučková M, Labuda J, Gründler P. Voltammetric detection of antioxidative properties of flavonoids using electrically heated DNA modified carbon paste electrode. *Sensors*. 2003 Jan 24;3(1):1-0.
[21] Barmana AK, Murshida GM, Saifuzzamana M, Mahaldara K, Bojićc M, Biswasa NN. Study of antioxidant potential, and quantification of major polyphenols in Leonurus sibiricus L. leaves using HPLC. *Pharmacol*. 2017 Apr 30;17:55-67.
[22] Patel RM, Patel SK. Cytotoxic activity of methanolic extract of Artocarpus heterophyllus against A549, Hela and MCF-7 cell lines. *Journal of Applied Pharmaceutical Science*. 2011 Sep 30(Issue):167-71.
[23] Grever MC, Chabner BA. Cancer drug discovery and development. *Cancer: Principles and Practice of Oncology*. 2001;1.
[24] Islam MS, Al Mansur MA, Rakhi SA, Sarkar MR, Kuddus MR, Ahmed F. Antioxidant, thrombolytic, cytotoxic and antibacterial activities of leaves of Vitex peduncularis. *Bangladesh Journal of Scientific and Industrial Research*. 2022 Dec 25;57(4):239-46.