A STUDY ON THE EFFECT OF ETHYL ACETATE EXTRACT OF *TERMINALIA BELLIRICA* FRUIT AGAINST OVARIAN CANCER CELLS.

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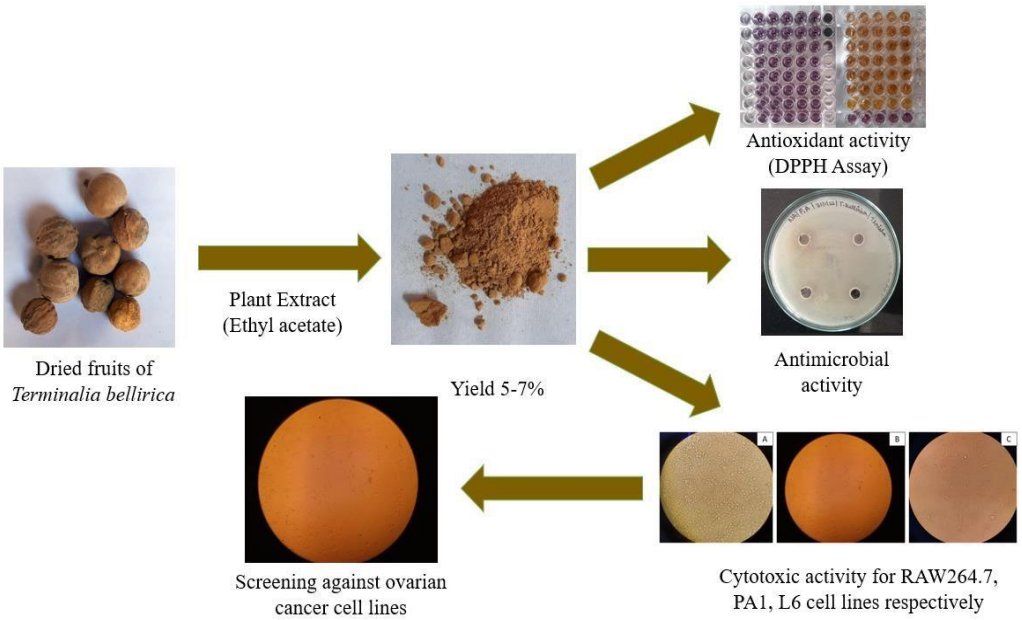
ABSTRACT:
Terminalia bellirica is one of the most planted and utilized as traditional medicine around South-East Asia. The various conventional medical systems, including Ayurveda, Unani, Siddha, and traditional Chinese medicine, have each highlighted the benefits of fruits of this plant. The fruits of this plant contain glucoside, tannins, gallic acid, ethyl gallate, and chebulinic acid, which have anti-inflammatory, anti-cancer, antidiarrheal, anti-microbial, and antipyretic properties. Yet scientific evidence-based molecular mechanisms have not been fully reported. This present study has highlighted the antioxidative, antimicrobial, and anticancer properties of the ethyl acetate extract of *Terminalia bellirica*. Antibacterial and antifungal activity was carried out against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Saccharomyces cerevisiae, and Candida krusei* respectively. Cytotoxicity studies were carried out for cell lines such as RAW 264.7, L6, and PA1. The results of this study demonstrate excellent efficacy against PA1 (ovarian cancer) cell lines.

Keywords: *Terminalia bellirica*, traditional medicines, ethyl acetate extract, PA1 cell line.



Graphical abstract

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INTRODUCTION:

The plant world is a veritable pharmacy of herbal remedies. Natural goods made from plants are an essential source of prosperity for a nation because they are used as the basis for the creation of medicines [1,2]. Bioactive substances taken from natural sources have been used as targets or templates for the therapy of different illnesses. A World Health Organization report states that the traditional medical system serves the basic healthcare requirements of about 80% of the people in developing nations [3,4]. One such plant is *Terminalia bellirica* Roxb, which has about 200 varieties and is the second-largest genus in the family Combretaceae after Combretum. Given that the leaves are organized at the ends of the stalks, the Latin word "terminus" is where the name "Genus Terminalia" comes from [5]. The plants in this family are found throughout tropical areas such as India, Nepal, Sri Lanka, and Southeast Asia, with South-East Asia having the highest genetic variety. The regional names for Terminalia bellirica include "Bahera" in Hindi, "Beleric Myrobalan" in English, "Bibhitaki" in Sanskrit, "Tanrikkai" in Tamil, "Tannikai" in Malayalam, and "Tarekayi" in Kannada [6].

In addition to its truncated trunk, which has superficial longitudinal fissures, it has a brownish-grey bark and reaches a height of 20 to 30 meters. The alternate arrangement, flawless edges, rounded ends or subacute, elliptical obovate, distinct midrib, hairy when young, and becoming hairless with age characterize the leaves of this plant. The blooms are axillary racemes that are smaller than the leaves and longer than the petioles. They are a light greenish-yellow color and have a disagreeable smell. The fruit is an ovoid drupe that is originally rosy and round before drying out to become five-sided and brownish. An ovoid germ is present in every fruit. The epidermis is grey or brown with horizontal cracks and shallow breaks [7].

Terminalia bellirica is linked to the diverse biological actions of its multiple bioactive secondary metabolites, which include alkaloids, flavonoids, lignans, tannins, phenols, coumarin, terpenoids, glycosides, and saponins that contribute to ethnomedicinal attributes for various herbal formulations [8]. Furthermore, several methods, including high-performance liquid chromatography, ultraviolet-visible spectrophotometry, infrared spectroscopy, and gas chromatography-mass spectrometry (GC-MS), have been used to identify and characterize bioactive compounds [9,10,11]. On a dry weight basis, 12.28% of the seed's weight was made up of oil. According to reports, seed oil includes stearic acid (16%), palmitic acid (12%), linoleic acid (29%), and oleic acid (43%) [9]. Per 100 g of seed, the seed kernel contains 22.57% total fat, 8.38% total protein, and 19 mg, 0.45 mg, 0.79 g, and 1.1 mg of vitamin B1, B2, C, and A, respectively.

Fruits of this tree are well known to help with inflammatory properties of lungs i.e., asthma, bronchitis and liver i.e., hepatitis. They are also well known to cure indigestion haemorrhoids, dysentery, growling voice, scorpion stings and best-known hair tonic [10,11]. The green fruit's decoction is used to treat coughs [7]. Fruit that is just partially ripe is used to purge. The triterpenoid chemicals found in fruits have been linked to significant antibacterial activity [3]. In Khagrachari, fruits are used to treat menstruation irregularities [2]. Rheumatism is treated with seed oil [13]. The leaves also strengthen the body's ability to fend off viruses, promote immunity, raise appetite, reduce piles, lower cholesterol and blood pressure, and boost blood flow [16]. Phyllanthus emblica L. (also known as Emblica officinalis Gaertn.), T. chebula Retz., and T. bellirica, three plant species endemic to the Indian subcontinent, are the ingredients of "Triphala," a well-known natural remedy in Ayurveda medicine. As it preserves the balance of three essential physiological bioelements, or "doshas," such as "Vata" (properties of dry, cold, light, minute, and movement), "Pitta" (metabolism), and "Kapha," it is referred to as a "tridoshic rasayana" in Ayurveda (watery element). "Triphala" encourages rebirth and longevity in people of all ages [17].

MATERIALS AND METHODS:

Diphenyl Picryl Hydrazyl (DPPH) radical Scavenging Method:

The DPPH radical scavenging test is used to evaluate the antioxidant activity. This test's technique is based on measuring how well antioxidants can scavenge stable radicals (DPPH). The DPPH free radical is converted to the equivalent hydrazine when it interacts with hydrogen donors; this ability is measured using the discoloration assay, which measures the absorbance decrease at 518-528nm caused by the addition of the antioxidant to a DPPH solution in methanol.

The 96-well microtitre plate was used for the test. 10µl of each test sample was introduced individually to 200µl of the DPPH solution in the microtitre plate wells. The microtitre plate was incubated for 20 minutes at 37°C to measure the absorbance using an ELISA reader at 490nm. The proportion of the material needed to scavenge 50%

of DPPH free radicals is known as the inhibitory concentration or IC50.
IC50 is calculated as follows: ((Control – Sample)/Control) *100 [18,19].

Anti-microbial activity:
Three bacterial strains, including *Staphylococcus aureus*, a Gram-positive bacterium, *Pseudomonas aeruginosa*, *Escherichia coli*, a Gram-negative bacterium, and two fungus isolates, including *Saccharomyces cerevisiae* and *Candida krusei*, were used in the study. Based on their therapeutic and pharmacological significance, they were chosen.

The same amount of medium inoculated with the test organism (100 l) was made in a set of Petri dishes, and each of them was prepared (9 mg in 3 ml). If the drug concentration in the first tube is 6000 g/ml, the second tube will have a drug concentration of 3000 g/ml, the third will have a drug concentration of 1500 g/ml, and so on. Usually, a stepwise dilution by a factor of 2 (two-fold serial dilution) was used. One tube was left empty to act as a positive control for organism growth. (Weighed at 6mg/ml). The cultures were kept for the recommended amount of time (one day at 37 °C) at the ideal setting for the development [20,21].

Cytotoxicity:
RAW 264.7
A semi-adherent macrophage-like cell line known as RAW264.7 was developed from BALB/c mice that had been infected with the Abelson leukaemia virus. For the study of cellular responses to microorganisms and their products, these cells are frequently utilised as a model of mouse macrophages. This cell line's popularity is a result of its quick proliferative rate and straightforward handling. The doubling time for this cell line ranges from 11 to 30 hours. When RAW264.7 cells are adhered to the surface of the culture dish, which typically takes around 24 hours, the maintenance phase of the cells' growth begins. Every two to three days, but no more than four days, the growth media should be replaced. RAW264.7 cells are typically between 10 and 20 microns in diameter. It is possible to polarise RAW264.7 M0 macrophages into M1 or M2 macrophages. Moreover, pinocytosis and phagocytosis, which are vital tasks of macrophages in the body, are processes that RAW264.7 cells are capable of doing. It's vital to remember that RAW264.7 cells are an immortal cancer cell line. They participate in controlling bone remodelling even though they are not osteoclasts [22].
For passaging of the cell lines were removed from the medium and PBS was used to devoid of calcium and magnesium to rinse the adhering cells (3-5 ml PBS for T25, 5-10ml for T75 cell culture flasks). It is necessary to completely cover the cell sheet before adding Accutase (1-2ml per T25, 2.5ml per T75 cell culture flask). 10 minutes should be spent incubating at room temperature. Gently resuspend the cells in 10 ml of medium, centrifuge for 3 min. at 300 g, resuspend the cells in new medium, and then transfer the cells to new flasks that are filled with new medium [23].

PA 1
By using cells extracted from ascitic fluid, the line was created. When the serum concentration is low, the plating density is low, or the cells are exposed to 5-bromo-2'-deoxyuridine, the cells differentiate to produce embryoid bodies. F9 cannot be seen, although the embryonic antigen PCC4 is expressed[24].
For passaging of the cell lines were removed from the medium and PBS was used to devoid of calcium and magnesium to rinse the adhering cells (3-5 ml PBS for T25, 5-10ml for T75 cell culture flasks). Before adding TrypleExpress, make sure the cell sheet is completely covered (1-2ml per T25, 2.5ml per T75 cell culture flask). 10 minutes should be spent incubating at room temperature. Gently resuspend the cells in 10 ml of medium, centrifuge for 3 min. at 300 g, resuspend the cells in new medium, and then transfer the cells to new flasks that are filled with new medium. 1:4 is the split ratio[23].

L6
The rat L6 myoblast cell line proved a better model for the research of thyroid hormone (TH) (Koenig and Smith1985). L6 myoblast-derived cells were therefore kept at 37 °C, 5% CO2, 10% foetal bovine serum (FBS), and 100 g/ml of the antibiotic penicillin-streptomycin. To encourage the production of myotubes, the media was switched to DMEM containing 2% horse serum (HS) after 3 days of proliferation with DMEM containing 10% FBS [25].
For passaging of the cell lines were removed from the medium and PBS was used to devoid of calcium and magnesium to rinse the adhering cells (3-5 ml PBS for T25, 5-10ml for T75 cell culture flasks). Before adding TrypleExpress, make sure the cell sheet is completely covered (1-2ml per T25, 2.5ml per T75 cell culture flask). 10 minutes should be spent incubating at room temperature. Gently

resuspend the cells in 10 ml of medium, centrifuge for 3 min. at 300 g, resuspend the cells in new medium, and then transfer the cells to new flasks that are filled with new medium. 1:4 is the split ratio[23].

The L6, RAW 264.7, and PA 1 cell lines were harvested and enumerated in a hemacytometer. The cells ($0.01*10^6$/well) were seeded in a 96-well dish and cultured in a CO2 incubator at 37 °C in a 5% humid environment. The cells were treated with various concentrations of the plant extract (1000, 500, and 250 mg/mL) for a total of 24 hours; the tests were carried out in triplicate. 50 µl of MTT solution (5 mg/mL) was added to the 96 well plate after 24 hours of drug treatment, followed by 3 h of incubation. The resulting formazan crystals were dissolved in DMSO, and a microplate reader was used to detect the final solution's absorbance at 540 nm. Relative to untreated cells, the relative cell viability was represented as a percentage[26,27].

%Growth inhibition= 100- (Mean O.D. of Individual Test Group/Mean O.D. of Control Group) *100.

RESULTS AND DISCUSSION:

Plant extract collection:

Ethyl acetate extract of *Terminalia bellirica* used for this study were procured and authenticated from K Patel Phyto Extractions Private Limited, Gujarat, India.

Antioxidative activity:

By using the DPPH technique, *Terminalia bellirica' s* 50% scavenging activity was evaluated.

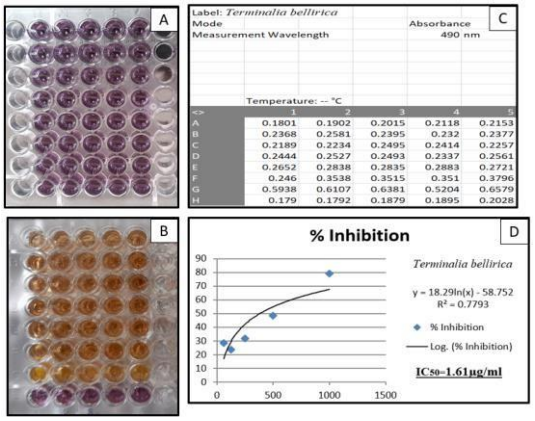

Figure 1: (A) 96 well plate containing DPPH and plant extract with concentrations 31.25, 62.5, 125, 250,
500, 1000, 2000, and methanol added as control. (B) 96 well plate after 30 minutes of incubation at 37
degrees Celsius. (C) Raw data at 490nm. (D) Graph and result in µg/ml

Anti-microbial activity:

Three bacterial species (Escherichia coli, Staphylococcus, and Pseudomonas aeruginosa) and two fungal species (Saccharomyces cerevisiae and Candida krusei) have been tested in vitro for the antimicrobial action of the plant product *Terminalia bellirica*. Highly substantial antibacterial action has been seen using the disc diffusion technique against evaluated pathogenic bacteria and fungi.

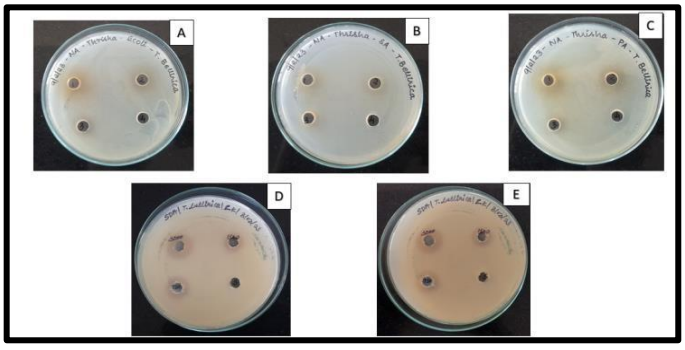

Figure 2: Zone of inhibition for the plant ***Terminalia bellirica*** against bacterial species such as ***Escherichia coli*** (A), ***Staphylococcus aureus*** (B), ***Pseudomonas aeruginosa*** (C), and fungal species such as ***Saccharomyces cerevisiae*** (D), ***Candida krusei*** (E). Concentrations are 3000µl (1), 1500µl (2), 750µl (3), Water (4)

Organism		**Zone of inhibition (cm)**		
	3000(µg/ml)	**1500(µg/ml)**	**750(µg/ml)**	**Water(µg/ml)**
Staphylococcus aureus	1.6	1.2	0.9	0.9
Pseudomonas aeruginosa	2.3	2	1	0.9
Escherichia coli	2.3	1.5	0.9	0.9

Table 1: Anti-bacterial activity results for *Terminalia bellirica*

Organism		**Zone of inhibition (cm)**		
	3000(µg/ml)	**1500(µg/ml)**	**750(µg/ml)**	**Water(µg/ml)**
Saccharomyces cerevisiae	1.9	1.4	1.3	0.9
Candida krusei	1.5	1.3	1.2	0.9

Table 2: Anti-fungal activity results for *Terminalia bellirica*

3.3 Cytotoxicity study:

By using the DPPH technique, *Terminalia bellirica's* CTC50 was evaluated.

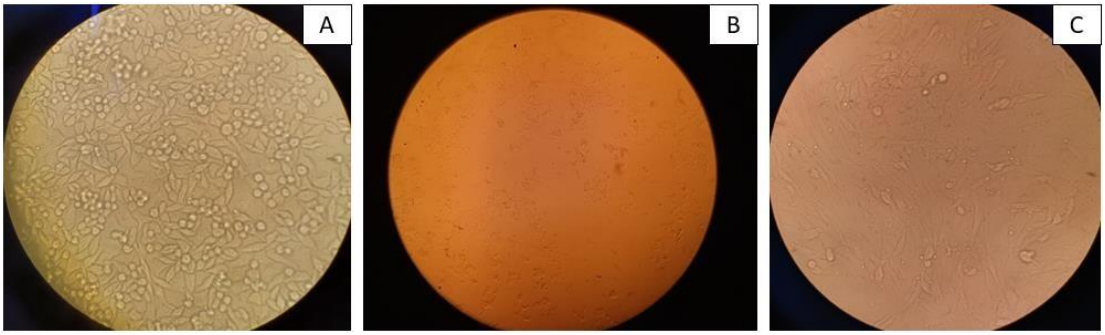

Figure 3: Photographs of cell lines such as (A) RAW 264.7, (B) PA 1, (C) L6

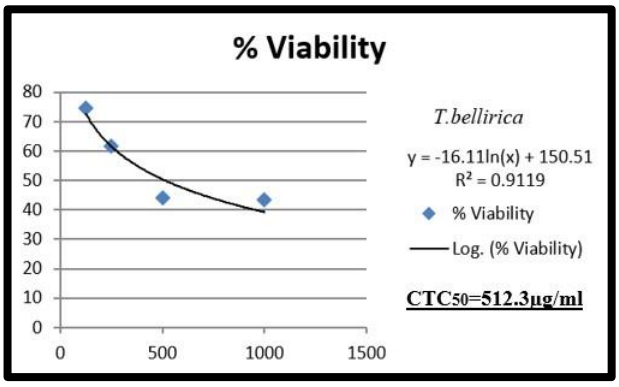

Figure 4: CTC50(Common Toxicity Criteria) of *Terminalia bellirica* against the L6 cell line

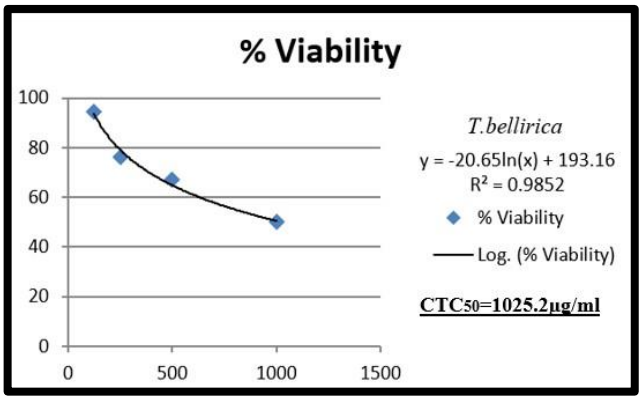

Figure 5: CTC50(Common Toxicity Criteria) of *Terminalia bellirica* against the RAW 264.7 cell line

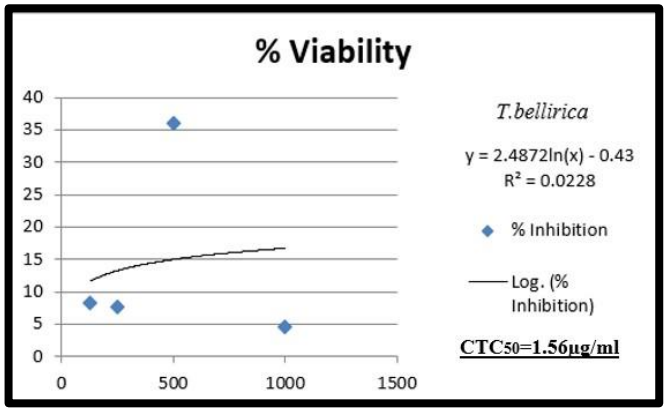

Figure 6: CTC50(Common Toxicity Criteria) of *Terminalia bellirica* against the PA1 cell line

Cell Type	Cell line used	Terminalia bellirica
Rat skeletal muscles	L6	512.33 µg/ml
Ovarian cancer	PA 1	1.5610 µg/ml
Monocytes	RAW 264.7	1025.26 µg/ml

Table 3: MTT assay results of *Terminalia bellirica*

CONCLUSION:

The given plant ethyl acetate extract of *Terminalia bellirica* was found to pose antioxidant, antimicrobial and cytotoxic activities. The cytotoxicity activity of *Terminalia bellirica* was found to be highly significant against ovarian cancer cell line (PA1). This study mainly focused on fundamental research related to the biological activity of *Terminalia bellirica*. Hence, further studies can be focused on anticancer activity against ovarian cancer cells and its molecular mechanisms. We are currently focusing on the mechanismbased studies related to *Terminalia bellirica*.

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REFERENCES:

1. Gupta A, Singh AK, Kumar R, et al, 2019. Corilagin in cancer: a critical evaluation of anticancer activities and molecular mechanisms. Molecules.24(18), Pages-3399.
2. Kumar N, Khurana SM, 2018. Phytochemistry and medicinal potential of the *Terminalia bellirica* Roxb. (Bahera). Indian Journal of Natural Products and Resources (IJNPR) [Formerly Natural Product Radiance (NPR)].9(2), Pages-97-107.
3. Kumar R, Singh AK, Gupta A, et al, 2019. Therapeutic potential of Aloe vera—A miracle gift of nature. Phytomedicine.60, Pages-152996.
4. Sharma AK, Sharma UK, Pandey AK, 2017. Protective effect of Bauhinia variegata leaf extracts against oxidative damage, cell proliferation and bacterial growth. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences.87, Pages-45-51.
5. Saxena VA, Mishra G, Saxena A, et al, 2013. A comparative study on quantitative estimation of tannins in *Terminalia chebula, Terminalia bellirica, Terminalia arjuna*, and Saraca indica using a spectrophotometer. Asian Journal of Pharmaceutical and Clinical Research.6(3), Pages-148-9.
6. Zhang XR, Kaunda JS, Zhu HT, et al, 2019. The genus Terminalia (Combretaceae): An ethnopharmacological, phytochemical and pharmacological review. Natural products and bioprospecting.9, Pages-357-92.
7. Deb A, Barua S, Das B, 2016. Pharmacological activities of Baheda (*Terminalia bellerica*): a review. Journal of pharmacognosy and phytochemistry.5(1), Pages-194-7.
8. Abraham A, Mathew L, Samuel S, 2014. Pharmacognostic studies of the fruits of *Terminalia bellirica* (Gaertn.) Roxb. J. Pharmacogn. Phytochem. 3, Pages-45-52.
9. Dhanani T, Shah S. and Kumar S, 2015. A validated high-performance liquid chromatography method for determination of tannin-related marker constituents gallic acid, corilagin, chebulagic acid, ellagic acid and chebulinic acid in four Terminalia species from

India. Journal of chromatographic science, *53*(4), pp.625-632.
10. Blainski A, Lopes GC, De Mello JC, 2013. Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from Limonium brasiliense L. Molecules.18(6), Pages6852-65.
11. Valli S, Shankar SG, 2013. *Terminalia bellerica*-a promising challenge to cryptococcosis. International Journal of Pharmaceutical Research and Bio-Science.2(5), Pages-154-69.
12. Pfundstein B, El Desouky SK, Hull WE, et al, 2010. Polyphenolic compounds in the fruits of Egyptian medicinal plants (*Terminalia bellerica, Terminalia chebula and Terminalia horrida*): characterization, quantitation and determination of antioxidant capacities. Phytochemistry.71(10), Pages-1132-48.
13. Singh A, 2011, editor. Herbalism, phytochemistry and ethnopharmacology. CRC Press.
14. Hazra K, 2019. Phytochemical investigation of Terminalia bellirica fruit inside. Asian J Pharm Clin Res.;12(8), Pages-191-4.
15. Mallik J, Das P, Karon B, et al, 2013. A review on phytochemistry and pharmacological activity of *Terminalia bellerica*. International journal of drug formulation and research;3(6), Pages-1-7.
16. Ghani, A, 2003. Medicinal plants of Bangladesh with chemical constituents and uses. 2nd edn. Asiatic Society of Bangladesh, Dhaka, Ramna.
17. Peterson CT, Denniston K, Chopra D, 2017. Therapeutic uses of triphala in ayurvedic medicine. The Journal of Alternative and Complementary Medicine.23(8), Pages-607-14.
18. Sánchez-Moreno C, 2002. Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food science and technology international.8(3), Pages-121-37.
19. Benkhaira N, Koraichi SI, Fikri-Benbrahim K, 2022. In vitro methods to study antioxidant and some biological activities of essential oils: a review. Biointerface Res. Appl. Chem. 12(3), Pages-3332.
20. Martinez J, Torres JM, Arteaga F, et al, 1978. Determination of the minimal inhibitory concentration of amphotericin B and miconazole for 21 strains of Aspergillus. Mycopathologia.64:147-51. Nandennavar V, Kulkarni P. ANTIMICROBIAL ACTIVITY OF JATIPATRADI CHURNA.
21. Soleimani Amiri S, Hossaini Z, Azizi Z, 2022. Synthesis and investigation of antioxidant and antimicrobial activity of new pyrazinopyrroloazepine derivatives using Fe3O4/CuO/ZnO@ MWCNT MNCs as organometallic nanocatalyst by new MCRs. Applied Organometallic Chemistry.36(4), Pages-e6573.
22. Taciak B, Białasek M, Braniewska A, et al 2018. Evaluation of phenotypic and functional stability of RAW 264.7 cell line through serial passages. PloS one.13(6), Pages-e0198943.
23. Masters JR, Stacey GN, 2007. Changing medium and passaging cell lines. Nature Protocols.;2(9), Pages-2276-84.
24. Chung WM, Chang WC, Chen L, et al 2013. MicroRNA-21 promotes the ovarian teratocarcinoma PA1 cell line by sustaining cancer stem/progenitor populations in vitro. Stem cell research & therapy.4(4), Pages-1-0.
25. LESMANA R, GOENAWAN H, TARAWAN VM, et al, 2018. Optimazing culture and differentiation L6 cell, C2C12 cell and primary myoblast cells culture. Cell Biology and Development.2(2).
26. Edmondson JM, Armstrong LS, Martinez AO, 1988. A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. Journal of tissue culture methods. 11, Pages-15-7.
27. Chen CH, Tsao YT, Yeh PT, et al, 2022. Detection of microorganisms in body fluids via MTT-PMS assay. Diagnostics.12(1), Pages-46.