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Unveiling the Therapeutic Potential of Methanolic Extract of *Amaranthus dubius*: Anticancer, Antibacterial and Antioxidant Activity

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Abstract

Many phytochemicals, present in plant, are very important for healing from different diseases. The seeds of ***Amaranthus dubius***, in particular has bioactive compounds which may have anti-cancer, anti-microbial and anti-oxidant functions. This study aimed to assess the anti-oxidant, anti-cancer activity of methanol extract of *A. dubius* leaf. We have isolated methanol extract from clean shed dried leaf of the *A. dubius* plant. We have used different concentrations of the extract and assessed the cell viability using MTT assay on L-132 lung cancer cell line, assessed the antibacterial activity through disc diffusion on human pathogenic bacteria, assessed antioxidant activity using DPPH scavenging action. Our results indicated that methanol extract of *A. dubius* plant leaf induced apoptosis of 70% L-132 cell at the concentration of 500 µg/ml. The extract showed best bacterial growth inhibition at 500mg/disc concentration against *Shigella boydii*. The extract also showed 66% DPPH scavenging activity where we have used ascorbic acid as positive control. In addition, endeavour to describe the antibacterial properties of the plant. Altogether, the methanol extract of the *A. dubius* plant leaf showed potential medicinal effects. In future, active compound(s) need to be isolated and introduce in animal model to see the best beneficial effect.

Keywords: *Amaranthus dubius*, Anticancer, Antioxidant, Antibacterial.

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Introduction

Phytochemicals are biologically active compounds found in plants and are now employed particularly in medicine and pharmacology for the discovery of new drugs and therapeutics(Mercy & Udo, 2018). As for the Indian traditional medicine systems, the two major systems are Ayurveda and Siddha together forming a predominantly herbal system of medicines. (G. Jyothi Reddy et al., 2023). According to the World Health Organization's survey, the majority of the population especially in the developing countries, ranging from sixty-five to eighty percent depends on plants for primary health care. This implies that the uses of plants in the management of diseases in traditional medicine due to their antimicrobial and antioxidant properties are very vital (Antibacterial, Antioxi Dant Activity of Ethanolic Plant Extracts of Some Convolvulus Species and Their DART-ToF-MS Profiling. Evid Based Complement Altern Med., n.d.). Specifically on the early medicines one could say that the roots of these early medicines can be found in the pharmacological, clinical and chemical investigation of every type of traditional medicine (Balunas, M.J., Kinghorn, 2005). For example, people in different societies across the globe have the belief that herbal medicine does not have side effects than 80% of the global population uses it (Butler, 2004).

Cancer is a disease in which cells grow in an uncontrolled manner, form lumps and are capable of invading other parts of the body. Lung cancer is a common form of cancer and the death rate caused by it is the highest in the world, especially due to the use of tobacco. Most of the bioactive compounds that have been discovered from natural products have been reported to possess anticancer properties at the cellular level mediating by immune response, apoptosis or autophagy, and cell cycle arrest. The currently available anticancer drugs and the plant-based compounds in the pipeline to be tested in the coming years in clinical trials are many. Some of the natural compounds that have been identified to have anticancer activities include Alkaloids, Flavonoids, Terpenoids, Polysaccharides and Saponins (Majumder et al., 2017).

In the current society, bacteria have enhanced their resistance to the synthetic and semi-synthetic antibacterial substances (Stanković et al., 2016). The available anti biotics also have side effects include; allergic reactions and immune suppression. Due to these complications and the increase in the susceptibilities of the bacteria to the available antimicrobial agents, the pharmaceutical industries have been faced with a big challenge of establishing new and effective drugs in eliminating the microorganisms and reducing the effects of the drug on the host organism. Natural sources of antimicrobial agents include plants used in traditional medicine, traditional healers and other medicinal resources (Kusuma et al., 2014).

Medicinal plants have been used in management of diseases and have been reported to pos-sess antioxidant activity. From the analysis, it has been postulated that the antioxidant proper-ty of these plant products is mainly due to phenolic compounds such as the flavonoids and phenolic acids, ascorbic acid, vitamin E and carotenoids (Sawadogo et al., 2006).

The plant Amaranthus dubius, which is a member of the Amaranthaceae, has those bioactive chemicals that are necessary for both its anti-cancer and anti-microbial and hepato-protective properties. The focus of the current study was on the leaf extract of A. dubius antibacterial, anticancer and anti-oxidant [DPPH].

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Materials and methods

Plant material:

Amaranthus dubius leaves were collected from Agartala and plant material has verified the authenticity of the plant by Professor Badal Kumar Datta, Taxonomy lab, Department of Botany, Tripura University.

Preparation of methanol Extract:

Fresh leaves were thoroughly cleaned with distilled water until all mud or debris were cleaned, and then shade dried. The leaves were ground into a fine powder by an electric blender. The powdered sample was packed and kept at -20 °C till further use. Next, 10 gm of the dry powder material mixed in 100 mL of methanol for extraction and was kept in orbital shaker for 72 hours at 120 rpm at room temperature. The mixture was filtered using Whatman filter paper 1 and filtrate was placed in petri plates to dry at 37°C incubator to allow the organic solvent to evaporate. Then the dried extract was scraped off from petri plates once the solvent had entirely evaporated and made stock solution dissolving in DMSO.

Maintenance of Cell line:

The L-132 cell line was used to study the anticancer property of this extract. The L-132 cells are the human Lung carcinoma cell line obtained from National centre for cell science (NCCS, Pune, Maharashtra, India) and grown at 37 °C in a humidified incubator with 5% CO_2 environment. The cells were cultured in DMEM (Dulbecco´s Modified Eagle Medium)**.**

Cell culture:

The cells line was culture primarily in a sterile culture plate in DMEM medium supplemented with 10% (v/v) heat-inactivated Fetal bovine serum (FBS, Gibco, Gaithersburg, USA), 1X antibiotic-antimycotic solution and 1X vitamin solution for overnight in 37ºC incubator with 5% CO_2 condition and allow the cells to be confluent.

Anti-cancer activity of methanol extract of *Amaranthus dubius* leaves:

The anticancer potential of the extract was investigated using the human lung cancer L-132 cell line. After having 80% confluent the L-132 cells were trypsinized and seeded in a 96 well plates where each well did possess 10^4 cells/well. After overnight culture cells were replaced with serum free media and incubated for 4 hours. Then cells were treated in presence or in absence of methanol extract of *Amaranthus dubius* at doses of 1 µg/ml, 100 µg/ml, and 500 µg/ml in triplicate for 24 h in a CO_2 incubator (37°C, 5% CO2). Following a 24-hour incubation period, each well received 10 µl of a 12-mM 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, which was then incubated at 37°C for an additional 4-h period. After 4 h incubation, 75µl of medium removed from each well without disrupting the formazan crystal. Finally, each well received 50µl of dimethyl sulfoxide (DMSO) to dissolve the formazan and the plate was then placed on orbital shaker for gently shaking for 10 minutes at room temperature. The absorbance was read at 540nm with a Synergy Hybrid plate reader. The following formula was used to calculate the viability percentage:

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$$\% \text{ of viability} = \frac{\text{Absorbance of sample} - \text{Absorbance blank}}{\text{Absorbance of Control} - \text{Absorbance of blank}} \times 100$$

Acridine orange/ethidium bromide dual staining method:
Morphological changes of apoptosis are also determined by Acridine orange / Ethidium bromide (AO/EtBr) staining of the cells. The L-132 cultured cells were plated on 6-well plate having coverslips and exposed to 500 μg/ml leaves extract for 24 h. Then cells were washed twice with PBS. The cells were stained with 0.5ml AO/EtBr for the next 5 minutes followed by wash in PBS solution for 3 times. The images were captured using a fluorescence microscope. AO/EtBr can stain normal cells because this dye can easily cross the cell membrane, and cells were green; apoptotic cells and apoptotic bodies which are derived from nuclear shrinkages, damages, blasting and blebbing were observed as orange-coloured bodies.

DAPI staining:
DAPI staining is to determine the number of nuclei and to evaluate gross cell morphology. It is a fluorescence dye that bound to double-stranded DNA which absorbs at 359 nm (ultraviolet light) and emits at 461 nm (fluorescent blue). Fluorescence intensity of bound DAPI is about 20-fold greater than that of free DAPI. Fluorescence intensity is thus linearly related to the amount of DNA present. The sample has been equilibrated with phosphate buffered saline (PBS). Next, approximately 300 μl of the diluted solution was added to the coverslip preparation, making sure that the cells are completely extended over the cells. Then the slide is allowed to incubate for 1.5minutes. After then Rinsing of the sample has been for several times in PBS and excess buffer has been drained off and mounted using a mounting medium with an anti-fade reagent. Using appropriate filters, the sample is seen under a fluorescence microscope.

Antibacterial activity

Microbial samples:
Four pathogenic bacterial strains including *Escherichia coli, Shigella boydii, and Staphylococcus aureus* were used for this purpose.
Preparation of Agar plate:
To study the anti-bacterial activity of this plant leaf extract, agar media is used for bacterial growth. In a conical flask, 14 gm of nutrient agar powder is dissolved in 500 ml of distilled water and autoclaved. Some blank petri dishes are also autoclaved according to requirement. Then inside the laminar flow chamber 20 ml sterile nutrient agar medium in liquid condition is poured in sterile condition into the 100 mm sterile petri dish. This nutrient agar is now allowed to solidify in the plate for 20-30 mins.
Preparation of Luria Britani Broth (LB Broth):
For *in vitro* culture of pathogenic strain, 25gm of LB broth powder is dissolved in 1000ml of distilled water and autoclaved.
Disc Diffusion Method:
In solid agar media in petridishes, 40μL of standardized suspension of test microorganisms

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were seeded into respective plates. The plates are incubated for 10mins at 37 ℃ incubator to dry after spreading. Different concentrations of methanol leaf extract of *A. dubius* (10 µg/disc, 100 µg/disc, and 500 µg/disc.) were prepared by reconstituting with DMSO (Dimethyl sulfoxide). The sterile blotting paper discs (5 mm in diameter & 0.4 mm in thickness) were then placed in the marked zones of Petri dish containing pathogenic strain in agar media. Then each of the different concentrations of extracts applied on each disc (5µL of leaf extract) of petri dish. DMSO was applied on the disc of control zone of petri dish instead of extract. The plates were incubated at 37ºC for overnight. The antibacterial activities were measured by zone of inhibition expressed in mm. All experiments were carried out in triplicate manner and the mean of the readings were recorded.

***Liquid culture method*:**

Each strain of microorganism was primarily cultured in three test tubes containing 3 ml LB broth medium with different concentration of methanolic leaf extract of *A. dubius* (100 µg/ml, 500µg/ml). The procedure was repeated on the test organisms using the standard antibiotic (**polymyxin B**) as positive control. A tube containing LB broth only was seeded with the test organism as described above to serve as negative control. All the culture tubes were then incubated at 37°C for 24 h. After incubation, the tubes were then examined for microbial growth by observing for turbidity. All experiments were carried out in triplicate and the mean of the readings were recorded.

Antioxidant activity:

The identification of antioxidants using (DPPH) 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity has been done according to Valco et al. 2007. The DPPH radical scavenging test is the most popular and sensitive antioxidant assay that is based on substrate polarity and hydrogen donation and or the ability to scavenge a free radical. DPPH is a stable purple colour, and it is available at 515 nm in methanol. This assay is in concordance with the fact that DPPH accepts a hydrogen (H) atom and reduces it to DPPH2 thereby changing colour from purple to yellow with a corresponding decrease in the absorbance at 515 nm among the scavenger molecules or the antioxidant. The colour change is measured and expressed in terms of absorbance values obtained with the help of spectrophotometer and used to calculate the parameter related to antioxidant activity. To make the stock solution, 2.4 mg of DPPH were dissolved in 10 ml of methanol. In a test tube, 3 mL DPPH workable solutions were combined with 100 µL of leaf extract. 3 ml of solution containing DPPH in 100 µL of methanol is taken as a control and ascorbic acid at a concentration of 10 mg/ml is used as a positive control. After that, the tubes were kept in complete darkness for 30 min. The absorbance was therefore determined at 517 nm. The following formula was used to calculate the antioxidant activity:

$$\%\text{ of antioxidant activity} = \frac{\text{OD of Control} - \text{OD of Standard}}{\text{OD of Control}} \times 100$$

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Results and discussion

Anti-Cancer Activity:

Acridine Orange/Ethidium bromide and DAPI assay: AO/EtBr is a green fluorescent dye having a low cell toxicity, and is sensitive to the chromatin state in cells, which is used to examine the nuclei change of apoptotic cells. The morphological changes of the apoptotic cells were determined by staining with both the AO/EtBr dye and DAPI to the treated and untreated L-132 cells. In Figure 1D, 500 µg/ml methanol extract of *A. dubius*, showed significant number of orange cells compared to control cells which are green colour. In figure 1B, significant number of cells contains fade DAPI stain which are apoptotic cells compared to control cells (blue colour cells in figure 1A).

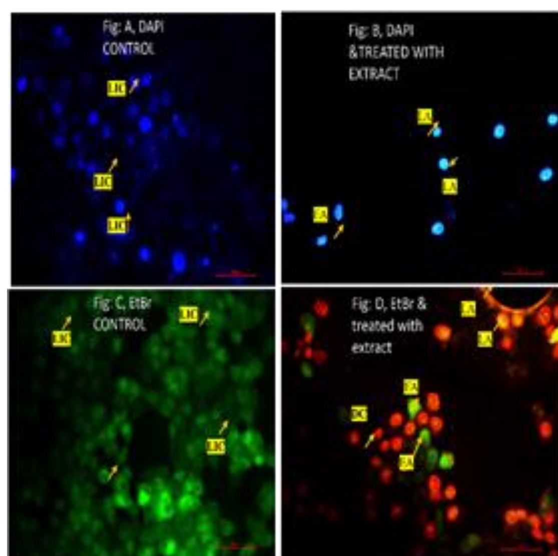

Fig 1: Apoptotic detection: Acridine Orange Ethidium bromide staining (AO/EtBr); DAPI Staining. *LIC: live intact cell; *EA: Early apoptotic body; *LA: Late apoptotic body; *DC: Dead cell

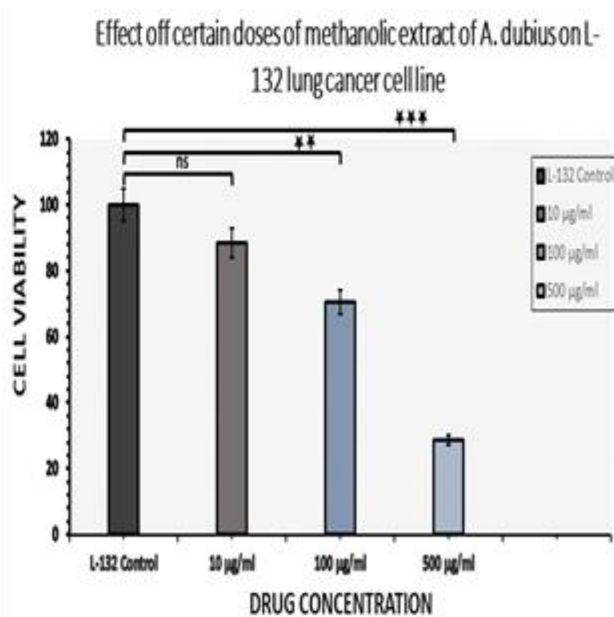

Fig 2: % of cell viability at different concentration of methanolic leaf extract of A. dubius Data represented as mean ±SD of three concentration of doses (*p<0.05, **p<0.01, ***p<0.001, ns- no significance p>0.05)

The MTT assay was used to determine the cell viability on effect of the leaf extract of *A. dubius* on the L-132 lung cancer cell line. MTT is a yellow tetrazolium salt that is taken up by cells via endocytosis. Within viable cells, MTT is reduced by mitochondrial dehydrogenases to form insoluble purple formazan crystals. In our experiment, after 24 hours of treatment, extract decreased the viable L-132 cells in a dose-dependent manner (10µg/ml, 100µg/ml and 500µg/ml). The highest effect was found at the dose of 500 µg/ml, where only 30% cell are viable compared to untreated control cells. Exactly which compound of methanolic leaf extract is responsible for this reduction of cell viability is not analysed in this study, but it is assumed that the presence bioactive compounds such as alkaloids, flavo-noids, tannins, quinones, glycosides, terpenoids, polysaccharides saponins in the methanolic leaf extract, have some

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effect in reduction of the viability of L-132 lung cancer cell line.

Antibacterial activity

We have tested the antibacterial activity of methanolic extract of *A. dubius* leaves on human pathogenic bacteria *Shigella boydii* , *E. coli* and *Staphylococcus aureus.* Using three different concentrations (10 µg/disc, 100µg/disc & 500µg/disc) in nutrient agar plate zone of inhibition were measured. *Shigella boydii* showed maximum zone of clearance at the dose of 500 µg/disc concentration of plant extract (Figure 3). Other two bacterial growth were not inhibited significantly (Figure 4).

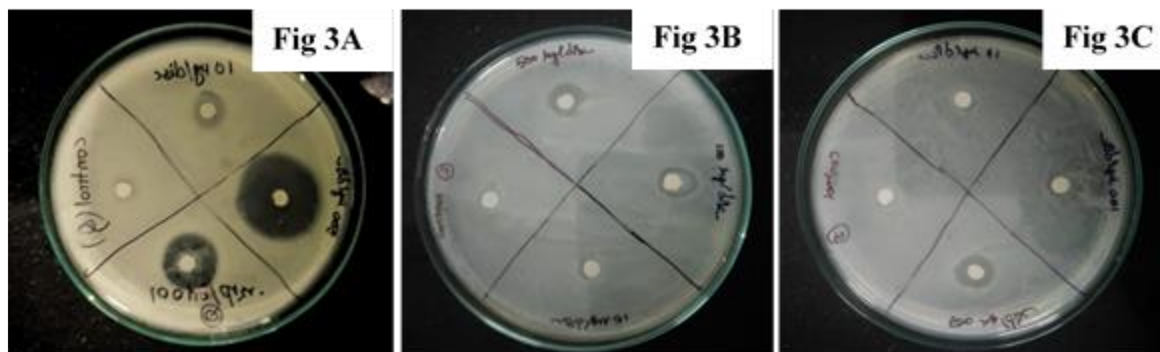

Fig 3: Effect of methanolic leaf extract of *A. dubius* on A. *Shigella boydii*; B. *E. coli;* C. *Staphylococcus aureus*

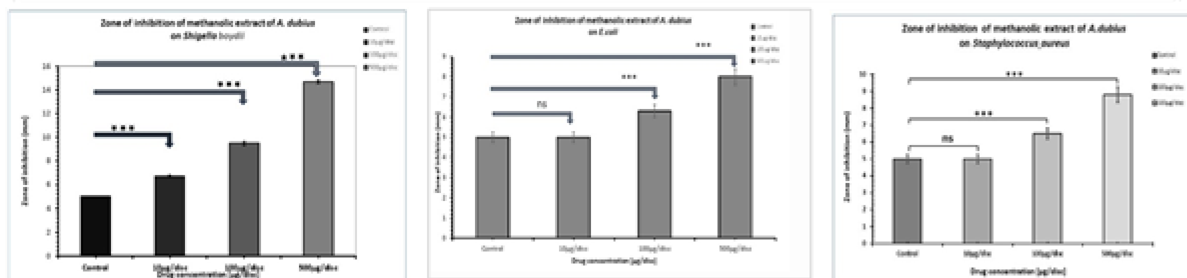

Fig 4: Bar diagram showing the effects of Methanolic leaf extract of *A. dubius* at different concentration against bacterial growth *(Shigella boydii, E. coli, Staphylococcus aureus)*. Data represented as mean ±SD of three concentration of doses (*p<0.05, **p<0.01, ***p<0.001, ns- no significance p>0.05).

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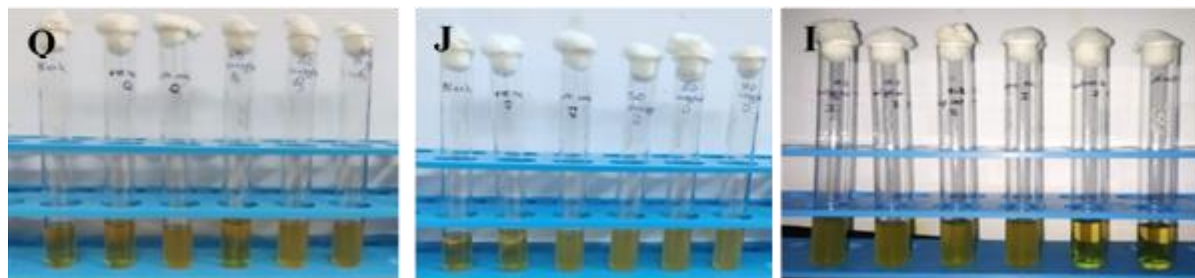

Fig 5: Effect of methanolic leaf extract of *A. dubius* on liquid culture of bacterial strains (Q= *Shigella boydii*; J= *E. coli;* I = *Staphylococcus aureus*) treated with different concentration of extract.

We have checked the growth inhibition of bacteria in broth culture method also. We have taken fresh cultured S. boydii, S. aureus and E. coli in nutrient broth and treated with different concentration (10 µg/ml, 100 µg/ml and 500 µg/ml) of extract. The result indicated that at the dose of 500mg/ml dose, the growth of S. bydii was completely inhibited ($p<0.001$) but the growth of other two bacteria were not inhibited completely at that dose. So, from these findings, it is confirmed that the methanolic leaf extract of *A. dubius* has anti-bacterial activity, but which compound is responsible for this activity is not known yet. Many studies have shown that secondary metabolites of plant extract like carotenoids and phenolic compounds and particularly flavonoids can target microbes through several mechanisms in a way which has been described as follows; they can inhibit nucleic acid synthesis that inhibit cytoplasmic membrane function, inhibit energy metabolism, and inhibit microbes from attaching and forming biofilm, bind to and block porin on the cell membrane, affect membrane permeability potentially leading to cell destruction in a way to reduce pathogenicity.

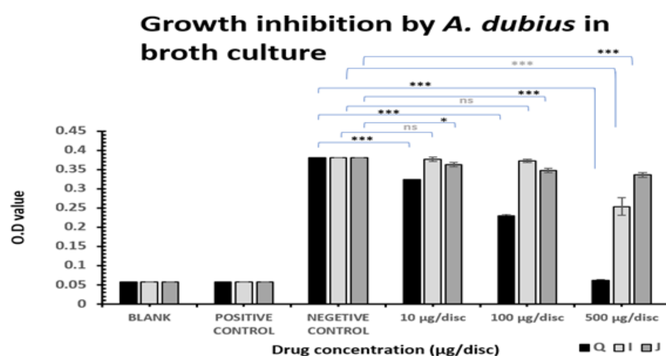

Fig 6: Bar diagram represent the experimental result of liquid culture of methanolic leaf extract of *A. dubius.* Data represented as mean ±SD of two concentration of doses (*p<0.05, **p<0.01, ***p<0.001, ns- no significance p>0.05).

Antioxidant activity:

Antioxidant activity of *A. dubius* was determined by using DPPH radical scavenging method. DPPH is a potent free radical compound which has been employed extensively to evaluate the free radical scavenging activity of different types of samples. In this study,

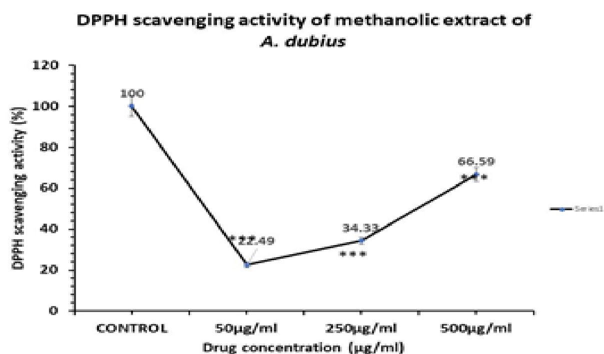

Fig 7: Line diagram represent the DPPH scavenging activity of methanolic extract of A. dubius. Data represented as mean ±SD of two concentration of doses (*p<0.05, **p<0.01, ***p<0.001, ns- no significance p>0.05).

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50, 250 and 500 µg/ml concentration were used to check the DPPH scavenging activity and ascorbic acid was used as positive control (Figure 7).

Result indicated that 500 µg/ml concentration of extract showed 66% DPPH scavenging activity compared to positive control. In lower concentration of the *A. dubius* plant extract had also some DPPH scavenging activity. Free radicals are generated in biological systems due to cellular metabolism and many other reasons. These free radicals can cause damage to tissues and biomolecules leading to various disease conditions, including degenerative diseases and severe tissue damage (Li & Beta, 2011). The reduction of absorbance of DPPH by antioxidants formed non-reactive stable compound through hydrogen donating process.

Conclusion:

From this study, it can be concluded that the methanolic leaf extract obtained from *A. dubius* has potential anti-cancer activity. This methanolic extract is able to produce significant cytotoxicity against L-132 lung cancer cell line causing apoptosis of cancer cell. This extract shows a significant anti-bacterial activity against *Shigella boydii* and has moderate growth inhibition effect on *Staphylococcus aureus* and *E. coli*. The presence of secondary metabolites like alkaloid, flavonoid, steroid, saponin, tannin, glycosides in the leaf extract may be responsible for its antimicrobial activity. The extract is also able to scavenge free radicals in biological system. In our experiment the extract shows highest scavenging activity that is 66.59% at the concentration of 500 µg/ml. In future, the active compound isolation from the leaf of *A. dubius* is required to check the best anticancer, antioxidant and antibacterial activities in *in vivo* system.

References:

Al-Rifai, A. A., Aqel, A., Al-Warhi, T., Wabaidur, S. M., Al-Othman, Z. A and Badjah-Hadj-Ahmed, A. Y. (2017): Antibacterial, antioxidant activity of ethanolic plant extracts of some Convolvulus species and their DART-ToF-MS profiling, eCAM. 1:1-9. https://doi.org/10.1155/2017/5694305

Balunas, M.J and Kinghorn, A. D. (2005): Drug discovery from medicinal plants. Life Sciences, Life Sci. 78(5): 431–441. https://doi.org/10.1016/j.lfs.2005.09.012

Butler, M. S. (2004). The role of natural product chemistry in drug discovery, J. Nat. Prod. 67(12):2141–2153. https://doi.org/10.1021/np040106y

Reddy, G. J., Madhavi, T. R., Rao, C. A., Nithya, S and Rao, P. N. (2023): An updated phytochemical and pharmacological review on Malabar Spinach *(Basella alba* or *Basella rubra*), GSCBPS. 24(2):161-169.

Li, W and Beta, T. (2011): Evaluation of antioxidant capacity and aroma quality of anthograin liqueur, Food Chem. 127(3):968–975. https://doi.org/10.1016/j.foodchem.2011.01.066

Majumder, D., Das, A and Saha, C. (2017): Catalase inhibition an anti cancer property of flavonoids: A kinetic and structural evaluation, Int. J. Biol. Macromol. 104:929–935. https://doi.org/10.1016/j.ijbiomac.2017.06.100

Mercy, E and Udo, D. (2018): Natural Products as Lead Bases for Drug Discovery and Development, Research and Reports of Medical Science. 2(1):1–2.

Sawadogo, W.R., Meda, A., Lamien, C.E., Kiendrebeogo, M., Guissou, I.P and Nacoulma, O.G. (2006): Phenolic content and antioxidant activity of six acanthaceae from Burkina Faso, J. Biol. Sci. 6(2):249–252. https://doi.org/10.3923/jbs.2006.249.252

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Stanković, N., Mihajilov-Krstev, T., Zlatković, B., Stankov-Jovanović, V., Mitić, V., Jović, J., Čomić, L., Kocić, B and Bernstein, N. (2016): Antibacterial and Antioxidant Activity of Traditional Medicinal Plants from the *Balkan Peninsula*, NJAS Wageningen J. Life Sci. 78: 21–28. https://doi.org/10.1016/j.njas.2015.12.006

Valko M., Leibfritz D., Moncol J., Cronin M.T., Mazur M and Telser J. (2007): Free radicals and antioxidants in normal physiological functions and human disease, Int. J. Biochem. Cell Biol. 39:44–84. https://doi: 10.1016/j.biocel.2006.07.001.

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