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Preventive Action of Blue Lotus (*Nymphaea caerulea*) Flower Extract against *E. coli*-Induced Immune-Pathological Changes In *Gallus gallus domesticus* Embryo

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Abstract

Nymphaea caerulea is an aquatic plant originally found in the Nile River has several therapeutic activities - anti-depressant, anti-inflammatory, anti-oxidant, and anti-microbial activities. This led us to perform the study focusing on the anti-microbial properties of the flower extract. Crude ethanolic extract of petals and pollens of *N.caerulea* flower were prepared and its antimicrobial activity was checked against *E.coli*. The Minimum Inhibition Concentration value was determined showing that both the extracts had similar capability against both the strains, with MIC values of 0.39mg/ml and 0.78mg/ml MIC against *E.coli* ATCC and MDR strains respectively. Further studies were done to study the gross morphological changes and the genetic expression changes of IL-10 and IFN-γ in *E. coli*-infected *Gallus gallus domesticus* embryo models. From the results, it can be said that the extract has preventive effects that reduce hemorrhages of the embryos when infected with *E.coli*. Moreover, there was a slight increase in the level of IL-10 cytokine gene expression indicating its anti-inflammatory action along with a higher increase in the IFN-γ cytokine gene expressions responsible for activating the host immunity. Thus, the findings indicate the probable potential role of *N.caerulea* flower extract to act as an antibacterial agent.

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Introduction

The world of medicine is evolving every day to fight against all the emerging diseases, and among them, one of the major concerns to biologists is combating Multi-Drug Resistance (MDR) pathogens. With injudicious use and easy availability of antibiotics, MDR pathogenic strains are now considered a serious problem worldwide. A very essential pathogen to be considered in this regard is *Escherichia coli*, a gram-negative, rod-shaped bacteria from the family Enterobacteriaceae. Responsible for inducing both intestinal and extraintestinal diseases in animals and humans globally with each pathotype displaying a very diverse realm of mechanisms of pathogenicity and virulence factors (Pakbin et al. 2021). Some common diseases caused by *E. coli* include community-acquired pneumonia (CAP), hospital-acquired

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pneumonia (HAP), bacteremia, urinary tract infections(UTIs), and several other nosocomial infections. Extended Spectrum Beta Lactamase (ESBL) are enzyme secreted by certain Enterobacteriaceae that causes resistance to a wide range of antibiotics. The major ESBL-producing bacterium is *E.coli*, this enzyme is known to degrade and annihilate the most used antibiotics, such as penicillin, cephalosporins, and aztreonam. ESBL is easily transmitted via plasmid thus making it resistant not limited to only beta-lactams but to also a wide range of other very frequently used antibiotics, namely, sulphonamides, aminoglycosides, and fluoroquinolones (Teklu et al. 2019). The phenomenon is responsible for making the antibiotics futile against infections and hence minimizing therapeutic choices.

The most effective treatment of choice to be considered now against pathogens producing ESBL is Carbapenems. However, in recent years carbapenem-resistant Enterobacteriaceae (CRE) strains have been frequently isolated due to their extensive usage, declaring it a "priority pathogen" by WHO for being the most potent threat to mankind (Pramanik et al. 2022). With more carbapenems being used against ESBL a new problem has arisen leading to the production of carbapenem-producing/Resistance Enterobacteriaceae (CPE or CRE). Carbapenemase includes mainly three major categories: Metallo-beta-lactamases (MBLs), for example; New Delhi MBL (NDM), *Klebsiella pneumonia* carbapenemase (KPC), and Oxacillinases (Sheu et al. 2019). To overcome this situation combinational drug therapy is being used, but as we observe new mutated and even stronger strains of pathogens with time, it is uncertain how long it will be effective. At the moment effective drugs against CRE include double carbapenem therapy, high dosage of tigecycline, and polymixins (colistin and polymyxin B). However, strains resistant to these antibiotics are rapidly emerging and thus there is a dire need for more effective antimicrobials to keep these imminent pathogens in check and provide a disease-free environment to the world (Bandy et al. 2021).

Herbal medicines have been in use since ancient times such as in Chinese traditional medicine and Ayurveda and are proven to be less toxic and have lower side effects than medicines made of chemicals. Medicines made from plant extracts are known to have an abundance of different bioactive compounds some may have antimicrobial activities, showing effectiveness against various microorganisms (Paudel et al. 2015). The problems caused by MDR pathogens have increased the search for new antibiotics from various sources which are both curative and at the same time not too costly. This has led scientists to show interest in herbal medicines and their antimicrobial nature. A very unique and assumed to be effective herbal therapy might be from the flower *Nymphaea caerulea* well known as Blue lotus or Egyptian lotus. A psychoactive plant whose origins are from the river Nile is believed to have many therapeutic uses, about which the Egyptian civilization was very aware. (Song et al.2021) Across India, there are nearly ten different species of *Nymphaea* discovered around Assam, Meghalaya, and Kerala. It is an aquatic plant found in abundance in rivers, lakes, and pools throughout Africa. The plant has immersed roots and stems, thus making it non-viviparous, The flower blue or violet consists of 4-5 sepals and 15-20 petals which have an angular shape and are long making the flower look like a star. The two main chemical compounds responsible for the flower's psychoactive nature are Apomorphine and Nuciferine, which is the source of its medical activity that ranges from antianxiety and antidepressant to anti-inflammatory and even aphrodisiac (Keppel et al. 2018). Some secondary metabolites of the plant such as flavonoids, saponins, alkaloids, phenols, glycosides, tannins, and many more are considered to have effective responses against various diseases (Saxena et al. 2021). Therefore, this leads to our experiment which focuses

on whether ethanolic extracts of the flower *Nymphaea caerulea* hold any antibacterial effects and thus can be developed into a potent antibiotic shortly. Recent scientific studies have established that Nymphaeaceae flower extract is effective against microbial pathogens (Akinjogunla et al. 2009) and (Dash et al. 2013). Hence there is no doubt that *Nymphaea caerulea* flower might hold some antibacterial nature that is yet to be highlighted and put to use. So, with the need to find new and effective antimicrobials against various pathogenic MDR strains, it could be a useful addition to the world of medicine and most importantly for the betterment of mankind.

Materials and Methods

Sample collection

Collection of plant sample: Freshly bloomed *Nymphaea caerulea* (Blue Lotus) flower (Figure. 1) was collected from the college garden of Heritage Institute of Technology, Kolkata, India on the 3rd of July, 2023 for obtaining the crude extract. The plant was harvested by a senior Botanist of the Institute who identified and collected the original plant.

Figure 1. *Nymphaea caerulea* (Blue Lotus) flower

Collection of bacterial strain: The two strains of bacteria used in this experiment, *Escherichia coli* ATCC 25922 and *Escherichia coli* MDR were collected from Peerless Hospital, Kolkata, India. The antibiogram of *E. coli* MDR is provided in Table 1.

Table 1. The antibiogram of *E.coli* MDR Organism selected: *Escherichia coli* (MDRO)

Antimicrobial	Mic	Interpretation	Antimicrobial	Mic	Interpretation
Meropenem	0.25	**S	Amoxicillin/ Clavulanic Acid	16	I
Amikacin	4	S	Piperacillin/ Tazobactum	>=128	R
Gentamicin	>=16	R	Cefuroxime	>=64	R

Ciprofloxacin	>=4	R	CefuroximeAxetil	>=64	R
+Levofloxacin	>=8	R	+Cefixime		R
Tigecycline	<=0.5	S	Ceftriaxone	>=64	R
Fosfomycin	>=256	R	Cefoperazone/ Sulbactam	16	S
Colistin	<=0.5	I	Cefepime	16	R
+Polymyxin B	<=0.5	I	+Doripenem	0.25	S
Trimethoprim/ Sulfamethoxazole	>=320	R	Ertapenem	<=0.12	S
			Imipenem	<=0.25	S
**= User modified					

Source: Urıne, Collected on July 21, 2023

Collection of chick eggs:The fertilized chick eggs *(Gallus gallus domesticus*) which were 14 days old were collected from the State Poultry Farm, Tollygunge, Kolkata, India. They were carried in an insulating thermocol box maintaining the temperature of around 38°C.

Preparation of crude ethanolic extract from blue lotus flower:

After collecting the flower, different parts of the flower; petals, and pollens were separated using clean forceps for making the ethanolic extractions of each part in three separate beakers. Each part of the flower was weighed separately and to each of them 70% Ethanol was added individually in the ratio of 1:10 (w/v) according to Table 2.

Table 2: Measurements of weight and volumes of each flower part to prepare the ethanolic extract

Part Of Blue Lotus Flower	Weight of flower parts (gm)	Volume of 70% ethanol (mL)
• Petal	0.7	7.0
• Stamen With Pollen	2.0	20.0

The beakers were then covered nicely with aluminum foil and kept in the dark for 72 hours for extraction to occur. Then extracts were passed through Whatman Filter paper over a funnel to new clean beakers. The filtrate was poured into three separate autoclaved ambered colored bottles via a Syringe filter inside the biosafety cabinet to avoid any contamination, the bottles were then stored in the refrigerator for further use (Prasad et al. 2016.).

Minimum inhibitory concentration (MIC) determination

ELISA (96 well) plates were taken to analyze the MIC values of *E.coli* ATCC 25922 and *E. coli* MDR respectively. To the first well of each column, 100µl of double-strength Mueller-Hinton broth was added and the rest of the wells were filled with single-strength Mueller-Hinton broth. Blue Lotus flower extract was now added 100µl in each well of the first row for three consecutive columns. This was carried out with Blue lotus petal ethanolic extract, Blue lotus pollen ethanolic extract, and 70% ethanol (control) individually.

Then serial double dilutions were done for every row. To each well 10µl of *E.coli* ATCC 25922 and *E.coli* MDR were given in two separate ELISA (96 well) plates. The O.D was then measured at 660 nm wavelength at 0 hours in an ELISA plate reader (Robonik readwell touch, India) and the plates were incubated for 24 hours at 37°C Again O.D was measured after 24 hours, and calculations were made after deductions of the first readings from final readings accordingly (Bussmann et al. 2010).

Preparation of egg (*Gallus gallus domesticus*) models for experimentation

Allantoic fluid collection and preservation:

The fertilized 14-day-old chick eggs (*Gallus gallus domesticus*) were nicely cleaned with distilled water after being brought to the laboratory. They were then candled with the help of a LED torch light to mark their air sacs and were incubated at 38°C maintaining a humidity of 60% - 80%. Before inoculation, the eggs were disinfected with 70% ethanol and iodine. They were then pricked with a sterilized needle in the air sac area marked before and the inoculum was injected accordingly. No inoculation in the normal control set, 100 µl of 70% ethanol in alcohol control sets, 100 µl of Blue Lotus petal (petal extract was used as in MIC results of petal extract and pollen extract were similar) ethanolic extract in drug control set, 10 µl of bacterial suspension of 0.5 McFarland standard in *E coli* ATCC control set, 100 µl of Blue Lotus petal ethanolic extract followed by 10µl of *E.coli* ATCC 25922 suspension of 0.5 McFarland standard after 1 hour of incubation at 38°C in Preventive challenge with the extract against *E.coli* ATCC 25922 set, and 10µl of *E.coli* ATCC 25922 suspension of 0.5 McFarland standard followed by 100 µl of Blue Lotus petal ethanolic extract after 1 hour of incubation at 38°C in the Curative challenge with the extract against *E.coli* ATCC 25922 sets.

All the sets were kept in the incubator at 38ºC for 5 hours. The eggs were then harvested with sterile scissors, forceps, and scalpels following basic bio-ethics. The allantoic fluid was collected into sterilized falcon tubes which were kept at -80ºC for further experimentation. (Das et al. 2022)

RNA extraction and RT-PCR

The RNA extraction procedure from the allantoic fluids collected was done according to the protocol provided with RNAiso plus manual kit (DSS Takara Bio India Private Limited, USA). The extracted RNA was then quantified in an UV-Vis Spectrophotometer to check the purity and quantity of the RNA extracted. Then cDNA was prepared by measuring appropriate volumes of RNA, Nuclease free water, and PCR Master mix with the cDNA Reverse Transcriptase Synthesis Kit from BIO-RAD.

To perform the gene expression studies of IL-10 and IFN-γ cytokines, reverse transcription-PCR was performed using the cDNA along with necessary primers (reverse and forward) specific to the cytokines to be studied and iTaqSyber green as the dying agent. Both the cytokine gene expressions were studied in comparison to a housekeeping gene β-actin in CFX-96 Instrument, USA, and RT-PCR (Bio-Rad, USA) (Goswami et al. 2022).

Blood film study

After ethically dissecting the chick (*Gallus gallus domesticus*) embryo blood was taken on a previously cleaned slide and a uniform smear was prepared with a spreader and allowed to dry completely. To these smears Leishman stain was added using a pipette to stain the film; overflow was avoided. This was kept for 2 minutes. Then distilled water (pH 7) was added dropwise using a pipette and mixed with the stain over the slide. After 10 mins the slide was placed under slow-running tap water for washing, the backside of the slide was then cleaned

with tissue paper and then allowed the slide to dry. The slides were then seen under the microscope (1000 x) and observation was done accordingly (Muhibi et al. 2019).

Statistical analysis

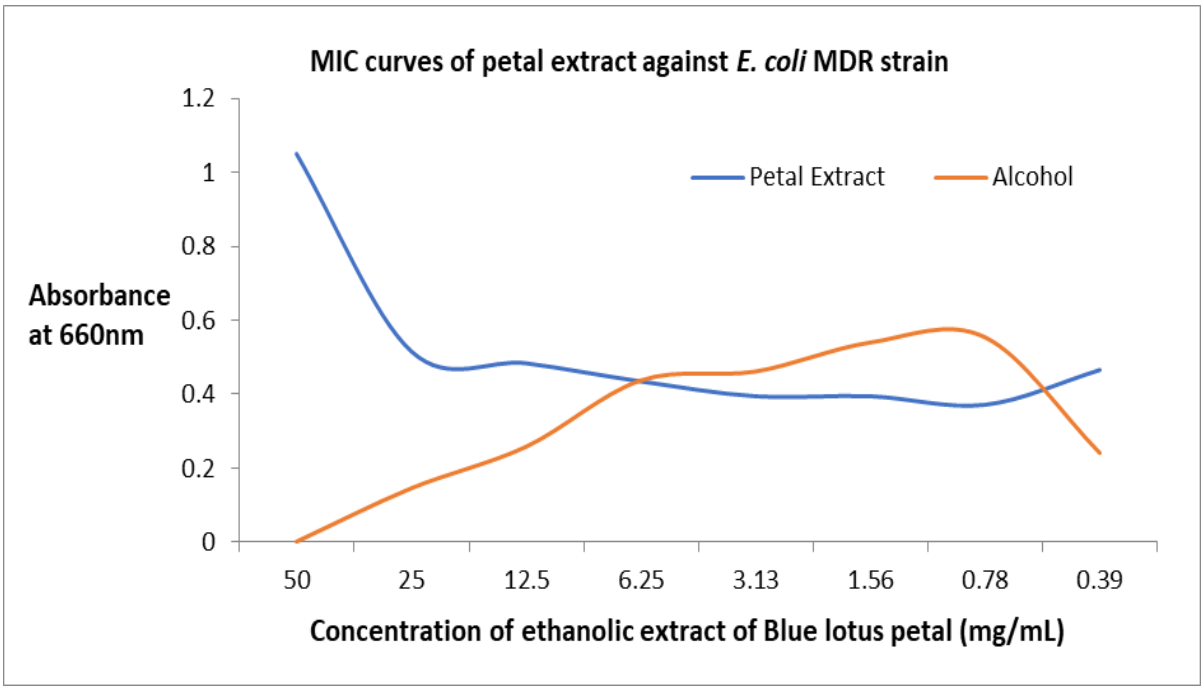
Statistical analysis was carried out with the help of Microsoft Excel 't' test analysis was done to ascertain the gene expression changes amongst different experimental sets and significance was considered at a level of 0.05 (P value).

Results and Discussion

A major concern in recent times in the field of medicine revolves around the strategies to find drugs that are both cost-effective and have potential antimicrobial effects on various MDR pathogenic bacterial strains. This threat has led us to perform this study using *Nymphaea caerulea* (Blue Lotus) flower extract and evaluate its antibacterial activities. Blue Lotus has been famously known to be used in oriental medicine and had various important roles during the Egyptian Civilization. Moreover, some recent scientific studies suggest the possible medicinal role of different wild species of *Nymphaea* (Pareek et al. 2016). Also, the phytochemical screening has shown the presence of various secondary metabolites which is of great importance to the antimicrobial nature of the flower extract. (Kebede et al. 2021).

Minimum Inhibitory Concentration (MIC)

MIC study of Blue Lotus petal extract and pollen extract against *E.coli* ATCC 25922 and *E.coli* MDR. Petal extract: The MIC value of the petal extract against *E.coli* ATCC 25922 was 0.39 mg/ml and MIC value against *E.coli* MDR was found to be 0.78 mg/ml. Thus, the MIC value is increased in MDR *E. coli* than in *E. coli* ATCC. The MIC curves are given in Figure 2.



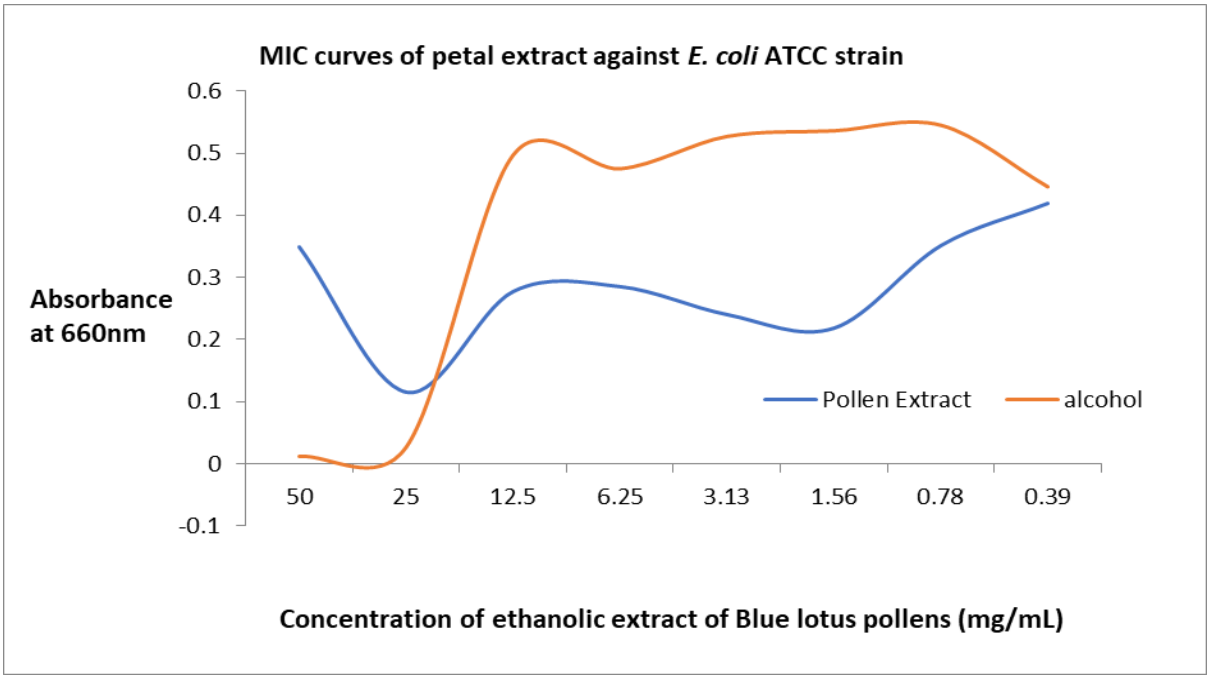
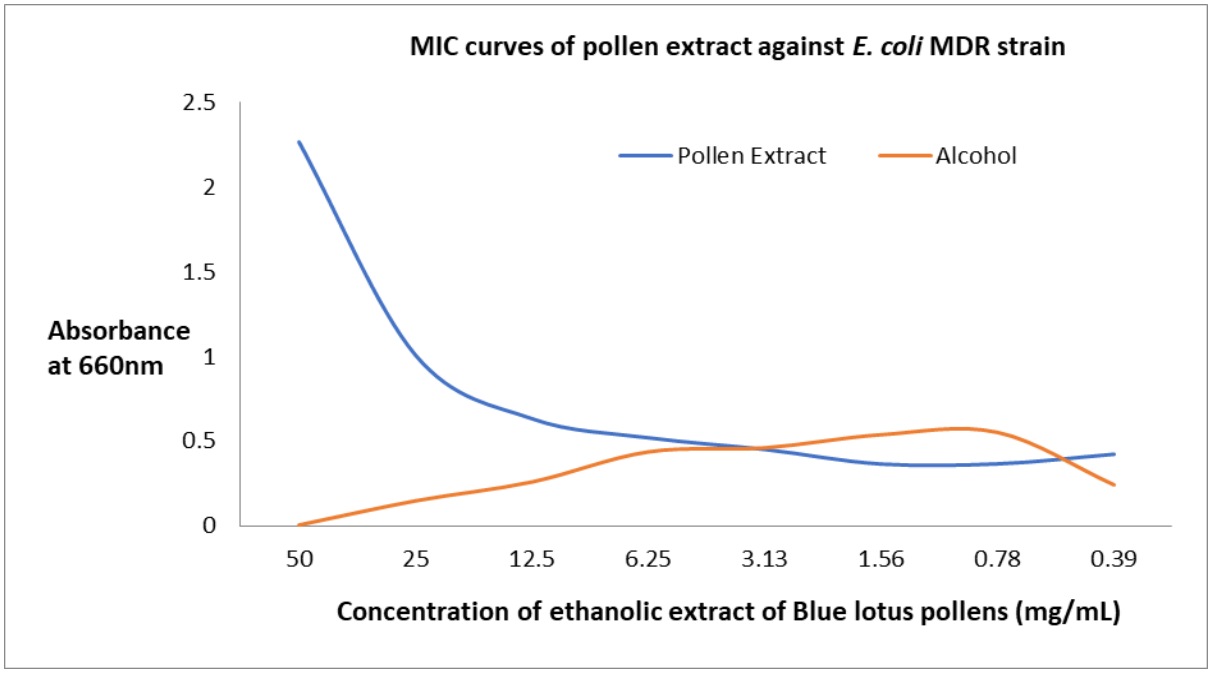


Figure 2: Graphical representation of MIC values of Blue Lotus petal extract against *E.coli* MDR strain

The high absorbance in higher concentrations is due to the color of the extract, The MIC value was 0.78 mg/ml. Graphical representation of MIC values of Blue Lotus petal extract against *E.coli* ATCC strain (Below). The high absorbance in higher concentrations is due to the color of the extract, The MIC value was 0.39 mg/ml. Stamen pollen extraction: Almost the same MIC values were also found with pollen extract against *E.coli* ATCC 25922 and *E.coli* MDR.

Thus, in relation to antimicrobial activities against *E.coli* ATCC 25922 and *E.coli* MDR both Petal extract and Pollen extract of the Blue Lotus acted in very similar ways. From the experimental result, it is clear that the ethanolic extract of both the petal and the pollen of the Blue Lotus flower shows a distinct Minimum Inhibitory Concentration (MIC) against both *E.coli* ATCC 25922 and *E.coli* MDR strains by both the petal extract and pollen extract had shown very similar MIC values thus proving that there is some antibacterial potential present in both the crude ethanolic extracts of the flower. The MIC curves of the pollen extract are given in Figure 3.



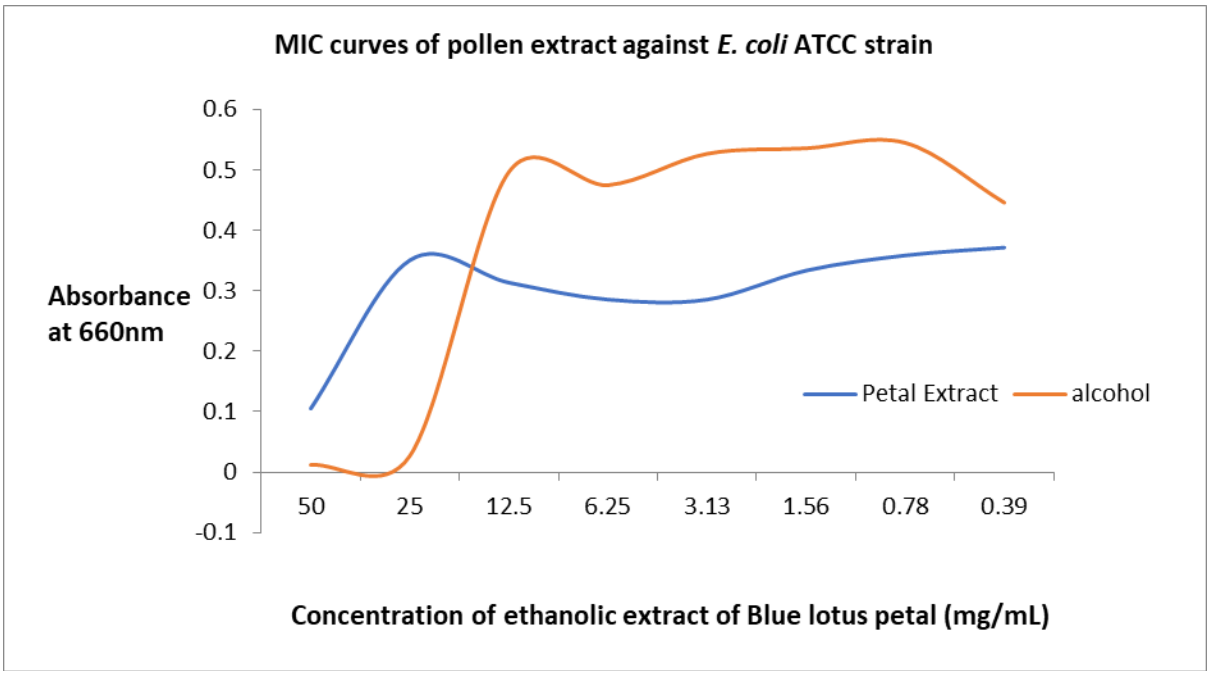


Figure 3. Graphical representation of MIC values of Blue Lotus pollen extract against *E.coli* MDR strain

The high absorbance in higher concentrations is due to the colour of the extract, The MIC value was 0.78 mg/ml. Graphical representation of MIC values of Blue Lotus pollen extract against *E.coli* ATCC strain (Below). The high absorbance in higher concentrations is due to the colour of the extract, The MIC value was 0.39 mg/ml.

Morbid anatomical study of *Gallus gallus domesticus* embryo in different experimental sets

Although the morphological pattern of the *Gallus gallus domesticus* embryos induced with 70 % ethanol appeared to be normal, there was mild to moderate degree hemorrhage observed. Almost similar findings were also observed in embryo models induced with Blue Lotus flower extract as well as in *E.coli* ATCC control and the curative set of experiments. On

the other hand, in the preventive set of the embryo model, the development of the fetus was excellent, and mild hemorrhage was observed. No other changes were observed in the morbid anatomical studies. The experiment was performed with *Gallus gallus domesticus* egg models, an emerging model to study epigenomics. Most importantly it has a homology to the human genome despite being only one-third of its size compared to the human genome and hence plays a crucial role in various biological studies especially in fields of immunology (Beacon et al. 2020) and (Gracia et al. 2021). It successfully specified that Blue Lotus flower extract has some explicit role in preventing pathogenic changes caused by *E.coli* ATCC 25922 only showing mild bleeding in the preventive set of our experiment; hence, demonstrating the ability to minimize the bacterial damage to the embryo. (Figure 4 A to F).

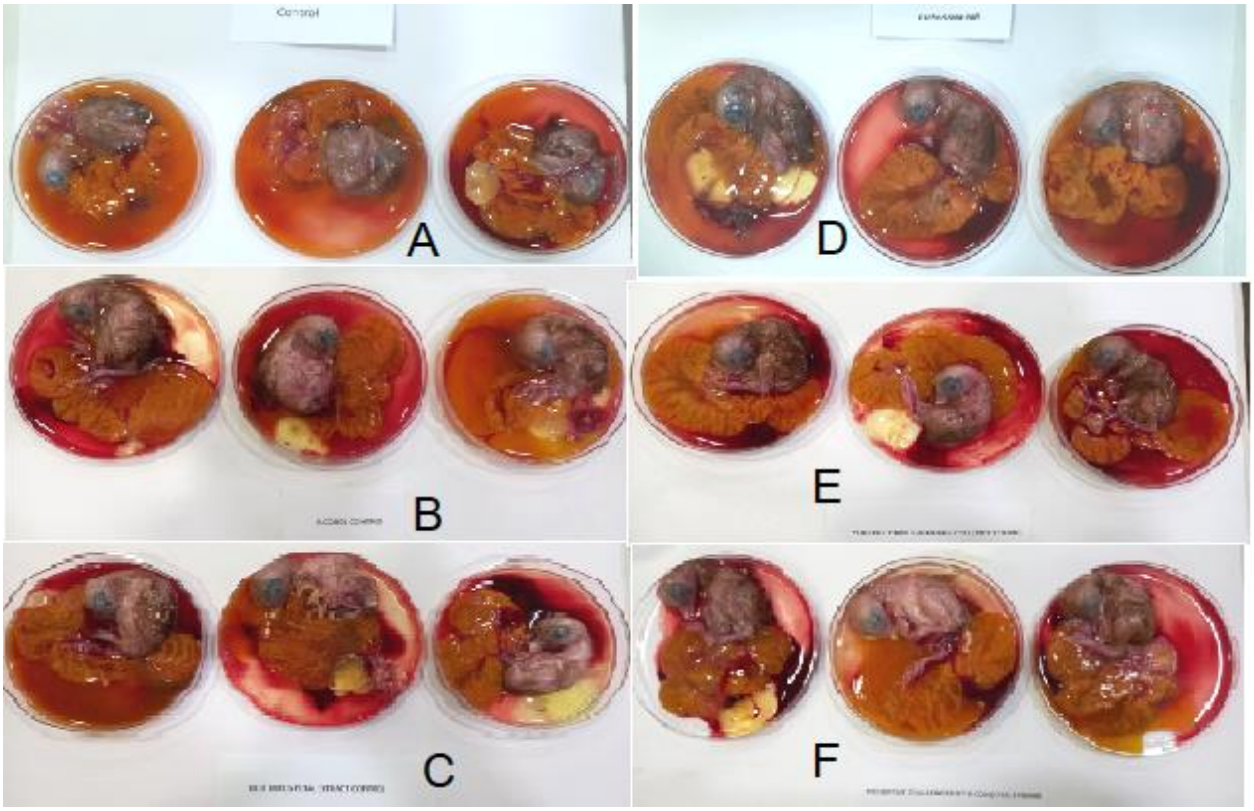


Figure 4: A: Normal control experiment set. B: 70% Ethanol control experiment set. C: Blue Lotus extract control experiment set. D: *E.coli* ATCC control experiment set. E: Curative challenge by *E.coli* ATCC experiment set. F: Preventive challenge by *E.coli* ATCC experiment set.

Cytokine gene expression changes in *Gallus gallus domesticus* embryo model

Changes in IL-10 gene expression: IL-10 gene expression was decreased in the curative set of experiments as well as in *E.coli* ATCC control and Blue Lotus extract control experimental sets. However, in the preventive and in 70% ethanol control experiment sets, IL-10 gene expression was increased. As IL-10 is a well-known anti-inflammatory cytokine, therefore the increase in gene expression observed in the preventive experimental sets might be beneficial to control the inflammation induced by *E.coli*.

Changes in IFN-γ gene expression: IFN-γ gene expression changes are highly significant in this experiment. Blue Lotus flower extract directly can increase IFN-γ gene expressions in a remarkable way (262.83 times). In the preventive experimental sets, the gene expression was further increased to 600.58 times which is an important biological action of the Blue Lotus

flower extract. However, in *E.coli* ATCC and the curative sets showed moderate increased levels of gene expression. The gene expression studies done with the main immune response regulators; IL-10 and IFN-γ gave significant information about the immune responses of the Blue Lotus flower extract. IL-10 is a well-known anti-inflammatory cytokine that acts when the host is infected by any microorganism, whereas IFN- γ is a type II interferon and activates macrophages. In general, it is a pro-inflammatory as well as anti-inflammatory cytokine and mainly helps activities related to Natural Killer cells and T-helper cells. Usually, IFN-γ is a cytokine that inhibits IL-10 expression, thus, IL-10 and IFN- γ are inversely related to each other. (Giunta et al. 2020; Wani et al. 2021). The experimental results clearly show an increase in IL-10 cytokine levels in the Preventive experimental set and the vehicle (70% ethanol) control sets as compared to the curative set-up, Therefore the Blue Lotus flower extract might be beneficial in controlling inflammation induced by the bacterium *E.coli* ATCC. However, the noteworthy increase in IFN- γ cytokine in all the experimental sets and specifically the preventive set indicates the ability of the Blue Lotus flower extract to initiate an antibacterial response by activating the innate immune system of the host (Figure 5). The IL10 gene expression changes in the Preventive experimental set between direct ATCC *E.coli*-induced gene expression and blue lotus preventive gene expression was significant at 0.0394 (P value) with a 't' value of 3.015.

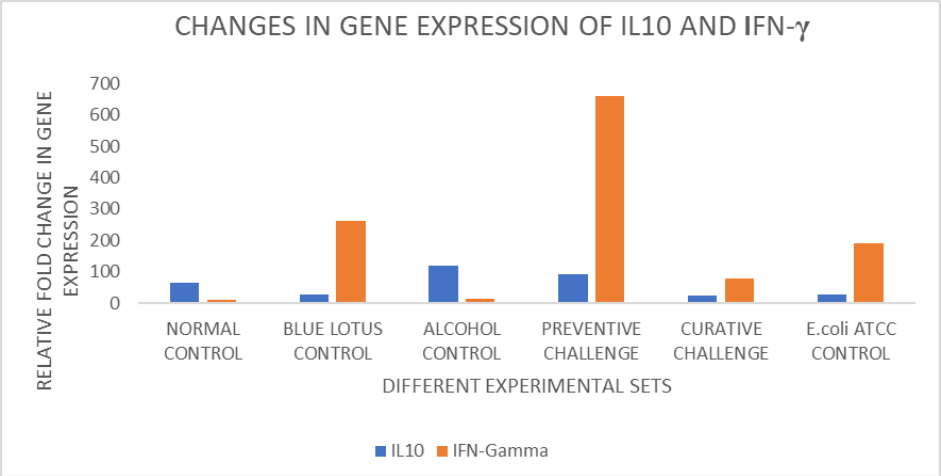


Figure 5. Graph showing changes in gene expression of IL-10 and IFN-γ. IFN-γ gene expression was significantly high in the preventive set although in the normal control, it was not up-regulated.

The action of blue lotus flower extract in *E. coli* induced hematological changes in the *Gallus gallus domesticus* embryo model

There are remarkable changes observed in the preventive action of Blue Lotus flower extract in *E.coli*-induced hematological alterations in the *Gallus gallus domesticus* embryo model.

The following changes were observed in each experimental model:

Control experiment with blue lotus flower extract: All the morphological appearances were normal, with the proper size, shape, and nuclear staining of the RBCs. Some senile RBCs were seen. The leucocyte count was mildly increased with predominant lymphocytes (more than 90%). An increase in the size of the lymphocytes was observed with an increase in the size of the nucleus. Chromatin of lymphocytes appears to be fine (Figure 6).

Control experiment with vehicle ethanol (70%): There were extensive morphological changes in RBCs. Although the size and shape of the RBCs were observed to be more or less normal, the size of the nucleus was markedly decreased and in most RBCs the nucleus is absent. Also, RBCs had non-uniform and distorted outlines and were mostly degenerated. The leucocytes were extremely rare as most have been damaged and only faint outlines of the lymphocytes were observed (Figure 6).

Curative experiment with blue lotus flower extract

The observation was almost similar to the 70% ethanol control set with slight improvement in RBCs and lymphocytes. The nuclei which were present in a few cells are faintly stained. An extremely lesser number of Lymphocytes were seen which can be considered to be almost absent. RBCs show more prominent outlines than in the case of the 70% ethanol control set (Figure 6).

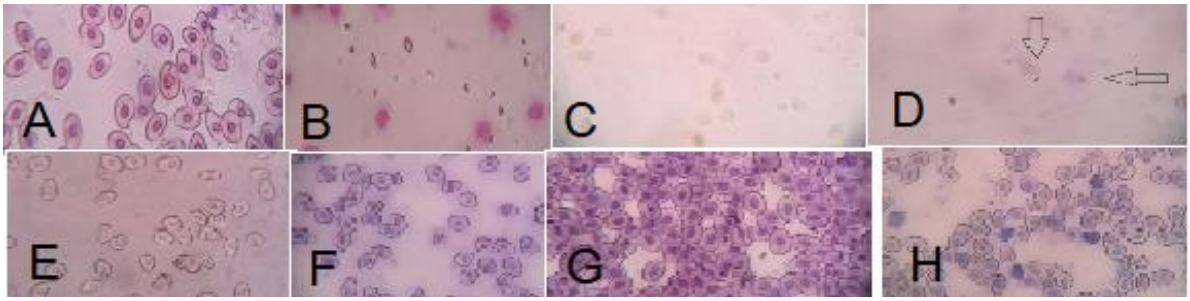


Figure 6. A. General appearance of RBC morphology in the normal control set. B. Morphological pattern of lymphocytes in the normal control set. C. Changes in RBC without nucleusand distorted outlines in the alcohol control set. D. Faintly stained outlines of lymphocytes in the alcohol control set. E. RBC changes in the curative set showing mild improvements. F.Normal RBCs in the preventive set. G.Crammed RBCs in the preventive set. H. Normal morphology of leucocytes with normal RBCs in the preventive set.

Preventive experiment with blue lotus flower extract

The morphology of all RBCs was observed to be excellent and was absolutely normal, with proper nuclei which were stained perfectly. The number of RBCs was increased and in some areas, they were almost crammed together. No senile RBCs were seen, all were of young generations. The lymphocytes were also morphologically excellent with clear outlines and properly stained nuclei (Figure 6).

E.coli as well as vehicle 70% ethanol can lead to extensive damage of the RBCs as well as the leucocytes. The number of RBCs and leucocytes was markedly decreased. In the curative experimental set, the Blue Lotus flower extract failed to revive all these changes; whereas, in the preventive experimental set the Blue Lotus flower extract excellently prevented all the adverse changes in RBCs as well as in leucocytes. The hematological studies done on *the Gallus gallus domesticus* embryo model showed that *E.coli* ATCC 25922 can cause damage to the RBCs and the leucocytes but when induced with Blue Lotus flower extract this damage can be prevented to a great extent. This was evident in the preventive sets of experiments. A possible explanation for this activity might be the regenerative power of the Blue Lotus flower extract which can have the power to stimulate bone marrow production

thus we can see all younger generations of RBCs in the preventive experiment set-up which is not seen in a set-up devoid of Blue Lotus extract.

Conclusion

The analysis results of the study help us conclude that there is a role of *Nymphaea caerulea* (Blue Lotus) flower extract in preventing pathogenetic changes caused by bacteria *E.coli* ATCC 25922. Thus, the ancient Egyptians probably not only used the flower as a psycho-promoting agent but also as a preventive drug against different microbial infections. Our experimentation result indicates its possible future usage as a potent drug in preventing different infections of the human race. With further proper study and correct dosage, Blue Lotus flower extract can be developed into an effective antimicrobial drug to fight against the worldwide concern of antibiotic resistance.

Acknowledgments

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Statement of ethics

For this study ethical approval was taken from the Institutional Ethics Committee although no ethical permission is necessary in experiments with less than 18 days old chick embryo. Ratification was obtained from the Institutional Ethical Committee (IEC) before the initiation of the research study on 22.07.2021.

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