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ANTIBACTERIAL ACTIVITY OF METHANOLIC EXTRACT OF FLOWERS OF *NYCTANTHES ARBOR-TRISTIS*

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Abstract

The synergistic inhibitory effect between conventional antibiotics and crude methanolic extract from the flowers of *Nyctanthes arbor-tristis L.* does not exist against *Pseudomonas aeruginosa* cultures.The essential oils are generally responsible for the fragrance of flowers. The flowers of *Nyctanthes arbor-tristis L.,* which are fragrant, was tested for its antimicrobial activity in this particular study. Crude methanolic extract from the flowers of *Nyctanthes arbor-tristis L.*is not an excellent material to conduct antimicrobial activity studied by agar well diffusion assay as the pigment Nyctanthin interferes with the visibility of zone of inhibition. Hydrodistillation by Clevenger is not an ideal method for isolation of essential oil from the flowers of *Nyctanthes arbor-tristis L.* Separation of Nyctanthin pigment from the active fractions will make the zone of inhibition more prominent. The synergistic inhibitory effect between conventional antibiotics and active fractions derived from the crude methanolic extract from the flowers of *Nyctanthes arbor-tristis L.* displayed a diffused zone of inhibition and hence is thought to have marginal antibacterial efficacy. The study demonstrated that the cold-press method for isolation of essential oil from the flowers of *Nyctanthes arbor-tristis L.* might be more effective an extraction procedure. Crude methanolic extract from the flowers

Keywords : *agar well diffusion assay, antimicrobial assay, essential oils, methanolic extract, Nyctanthes*

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of *Nyctanthes arbor-tristis L.* must be subjected to fractionation by column chromatography for proving its potency. The absence of synergism between methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* and conventional antibiotics could be possibly attributed to their different modes of action. It is not a complete surprise because the inhibitory effect of methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* alone at concentrations 9 µg, 18 µg and 25 µg was not discernible in terms of zone of inhibition in different strains of microbes like *Pseudomonas aeruginosa*, Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus. The negative results indicates that modificatons in extraction procedure is quintessential.

Introduction

Humans have been dependent on nature for curing various body disease. From ancient civilization, different plants were used to eradicate pain, control distress and thwart diseases. Most of the drugs used in primitive medicine were obtained from plants and are the earliest and principal natural source of medicines. The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming a significant cause of morbidity and mortality immune-compromised patients in developing countries, and many infectious microorganisms are resistant to synthetic drugs; hence an alternative therapy is very much needed(Phromnoi et al. 2011).Phytochemicals are widely used either as a single drug or combination in different health care systems. The plants used, as drugs are reasonably innocuous and relatively free from toxic effects or were so toxic that lethal effects were well known. Nature has provided the storehouse of remedies to cure all ailments of humanity.

Plants are reservoir of potentially useful chemical compounds which serve as drugs providing newer leads and clues for modern drug design by synthesis (Paul and Saxena 1997). *Nyctanthes abor-tritis* is known to provide solution for several ailments by tribal people of (Orissa and Bihar) India along with its use in Ayurveda,Siddha and Unani systems of medicines.Juice of the leaves function as digestives, an antidote to reptile venoms mild bitter tonic, laxative, diaphoretic and diuretic (Akki et al. 2009).Leaves extracts are also used in the enlargement of the spleen. Traditionally the powdered stem bark is given for treating rheumatic joint pain, in treatment of malaria and also used as an expectorant (Ratnasooriya et al. 2004).

The claimed traditional medicinal functions have been proved on a scientific basis using in-vitro and in- vivo experiments. The plants have been screened for an antihistaminic,analgesic,anti-inflammatory,amoebicidal,anthelmintic, antitrypanosomal, antidepressant, antiviral and immunomodulatory functions. Leaves extracts were found to have antimicrobial activity of the stem bark part.

Hence, the present study is aimed at the screening of the antimicrobial activity in the stem bark extracts of the plant Nyctanthesarbor-tristis L. Over 60% of the current anticancer drugs have their origin in one way or the other from natural sources. Dietary flavonoids and other polyphenols from medicinal plants are thought to have an essential role as chemopreventive agents. Nyctanthesarbor-tristisL. has been studied in great details for its anticancer properties. However, two iridoid glycosides Arbortristoside A and B have been reported to have anticancer activity against methyl-cholanthrene induced fibrosarcoma at 2.5 mg/kg in mice *Nyctanthes arbor-tristis* commonly known as 'night jasmine' or 'Harshringar' is an important medicinal plant of family Oleaceae widely used in Ayurveda. The most notable study which deals with this plant (Siriwardena and Arambewela,2014) focused on the determination of volatile constituents of the essential oil and absolute of Nyctanthesarbor- tristis flowers grown in Sri Lanka. This study led to the identification of 48 chemical constituents of essential oil through hydrodistillation extraction methods. The volatile sample was analyzed by GC- MS techniques.

The lion share of the essential oil extract was dominated by Phytol (32.2%) and methyl palmitate (17.4%).Other well known volatile constituents such as linalool, cucarvons, phytoene, nonadecane, methyl myristate, cis-9-tricosene, n-pentacosane, and geranylgeraniol were also identified in the essential oil. A similar study was followed through which shifted the focus on the plant specimens obtained exclusively from Dhaka city in Bangladesh. The white petal and orange corolla tubes of the flower, extracted by water distillation processing were subjected to GC-MS analysis. By this venture, the researcher obtained two major chemical constituents namely- 3,7,11,5-tetramethyl-2- hexadecane -1-ol (40.3%) and 2-methyloctadecance (17.6%) from petal and corolla tube (PriyaandGanjewala,2007). The flowers of *Nyctanthes arbor-tristisL.,* which are fragrant, was tested for its antimicrobial activity in this particular study. The essential oils are generally responsible for the fragrance of flowers. Hence in this study, we embarked to isolate the essential oil responsible for the fragrance of flowers of *Nyctanthes arbor-tristisL.* by subjecting the flowers to Clevenger extraction. Drug resistance in microbes is becoming a significant problem in the near future, and small molecules with antimicrobial potential are highly sought after. Flowers have been sources of antimicrobial agents (Tanojevic et al.2016). Since it was already reported that methanolic extract from leaves showed the maximum inhibitory activity against microbes, we subjected the flowers of Nyctanthes arbor-tristisL. to methanolic extraction,and this crude fraction was used to test its antimicrobial efficacy against microbes such as Klebsiella pneumoniae, *Pseudomonas aeruginosa,* Staphylococcus aureus and Escherichia coli. E. coli is a gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms (endotherms).Klebsiella pneumoniae is a gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium.*Pseudomonas aeruginosa* is a common encapsulated, Gram-negative, rod-shaped bacterium that can cause disease in plants and animals, including humans. Staphylococcus aureus is a Gram-positive, round-shapedbacterium that is a member of the Firmicutes, and it is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin. Pathogenic strains often promote infections by producing virulence factors such as potent proteintoxins, and the expression of a cell-surface protein that binds and inactivates antibodies. The emergence of antibiotic-resistant strains of S. aureus such as methicillin-resistant S. aureus (MRSA) is a global problem in clinical medicine.

Hence this study was initiated to isolate the essential oil responsible for the fragrance of Nyctanthes arbor-tristisL. flowers. Further, the extracts were evaluated for verifying their antimicrobial potential of methanolic extract of flowers of this plant.

Materials and Methods

Preparation of extracts from Nyctanthes flowers

500g fresh flowers were put in a round-bottomed flask of 500ml capacity. Then distilled water 50ml was added to it. The flask was connectedto the condenser of the Clevenger apparatus with silicone tubings. The round-bottomed flask was placed over the heating mantle. The temperature of the heating mantle was set at 40°C to prevent the thermal degradation of the essential oil in the flowers of Nyctanthes. Hydrodiffusion is the process of extracting the essential oil with steam that circulates through the plant material. At laboratory scale, we bring 50ml of water to heat and steam rises in a column. The vapour phase is then directed to a condenser and liquid is collected in a graduated burette. In that way, essential oil remains in the burette. After 2 hrs of extraction, calculated from the first condensed drop, we can measure the volume of oil recovered and calculate the yield from the mass of plant introduced. FreshNyctanthes flowers were collected and weighed (120g). Then they were immersed separately in a flask containing methanol (200ml). The lid of the flask was closed airtight to prevent the evaporation of methanol. The set up was kept at room temperature.

After three days, the solution was filtered, and the flower debris was removed. The extract was then evaporated using a hot air oven to yield a viscous mask. The crude extracts were weighed and diluted before being stored at 0 - 4°C for further analysis. Solvent extraction is a method to separate a compound into its parts based on the solubility of its parts. The flowers of *Nyctanthes arbor-tristis L.* were subjected to methanolic extraction. After the extraction, the debris was filtered using a sieve and filtrate was collected in a beaker. Later the filtrate was subjected to warm air drying, and the solvent methanol was evaporated. The concentrate was weighed and diluted in DMSO for further antimicrobial assays.

Determination of antimicrobial efficacy

Agar plates are inoculated with standardized inoculums of the test microorganisms separately. These included*Pseudomonas aeruginosa(*ATCC 27853), Escherichia coli(ATCC 25922), Klebsiella pneumoniae (ATCC 700603) andStaphylococcus aureus(ATCC 29213). Then, filter paper discs (about 6mm in diameter) containing the test compound at the desired concentration, were placed on the wells that created on the agar surface. The Petri dishes are incubated at 370C in a bacteriological incubator. Generally, antimicrobial agents diffuse into the agar and inhibit germinations and growth of the test microorganisms and then the diameters of inhibitions growth zones are measured (Balouiri et al.,2016).

Two different approaches were taken to determine the antibacterial property.

(1) The agarplates inoculated with bacterial strains are treated with determined concentrations of Nyctanthes arbor-tristis L.flower methanolic extract (9, 18, 25 ?g) and 10 % DMSO control, respectively). Then the Petri dishes were incubated at 370C in a bacteriological incubator.

(2) To see if the methanolic extract of Nyctanthes arbor-tristis L. flowers has an additive effect (Synergistic effect) along with other antibiotics on the cultures of Pseudomonas aeruginosa. Nyctanthes arbor-tristis L.flower methanolic extract at a concentration of 52 µg was added to the culture plate. Antibiotic discs containing antibiotics Cefotaxime 30 µg (CTX- 30), Cefixime 30 µg (CFM- 30), Cefixime 0.5 ?g (CFM- 0.5), Cefpodoxime 10 µg(CPD - 10) and Tetracyclin 30 µg (TE-30).

Results & Discussion

Fig. 1. *Nyctanthes arbor-tristis* a. Fresh flowers b. Flowers post extraction c. crude extract

The extraction of essential oil, which is the main constituent of fragrance in flowers and leaves of aromatic plants is by Clevenger apparatus or cold press method. Hydrodiffusion method by Clevenger of flowers of *Nyctanthes arbor-tristis L.* yielded only water-soluble fraction whose fragrance was different from that of fresh flowers. This may be because the essential oil was heat-labile and degrades even at a low temperature as low as 400 °C. Also, the solubility of the essential oil might be another reason for the absence of essential oil fraction in Clevenger apparatus. Since the extraction of essential oil from flowers of Nyctanthes arbor-tristis L. by Clevenger apparatus or hydrodistillation was unsuccessful, solvent extraction using methanol was adopted. The methanolic extraction procedure decolourized the flowers of Nyctanthes (Fig. 1b), and the Nyctanthin pigment was uniformly spread throughout the flower. The debris of flowers was also uniformly pigmented (Fig. 1b). The fraction of methanolic extract appeared dark red after the procedure (Fig. 1c). The extract was rich in pigments as it stained the hand and clothing which came in contact with it. The culture of E.coli a gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms was not affected by the methanolic extract of Nyctanthes arbor-tristis L. at the concentrations 9 µg (1), 18 µg (2) and 25µg (4) (Fig.3). The DMSO control at 10% did not affect the growth of E.coli reinforcing the negative control (Fig.2).

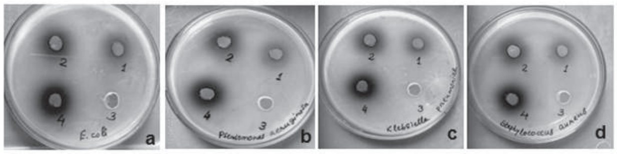

Fig. 2. Antimicrobial assay plates displaying the effects of *Nyctanthes arbor- tristis* on a. Escherichia coli b. Pseudomonas aeruginosa c. Klebsiella pneumoniae d. Staphylococcus aureus, wherethe numbers in plates stand for 9µg (1) , 18 µg (2), 25 µg (4) and 10% DMSO control (3)

The culture of P. aeruginosa, a common encapsulated, Gram-negative, rod-shaped bacterium that can cause disease in plants and animals, including humans was also not affected by the methanolic extract of Nyctanthes arbor-tristis L. at the concentrations 9 µg (1), 18 µg (2) and 25 µg (4) (Fig.2 a). Here too DMSO control at 10% did not affect the growth of P. aeruginosa reinforcing the negative control to be robust (Fig.2b). The culture of K. pneumoniae, a gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium was not affected by the methanolic extract of *Nyctanthes arbor-tristis L.* at the concentrations 9 µg (1), 18 µg (2) and 25 µg (4) (Fig.3). Here too DMSO control at 10% did not affect the growth of P. aeruginosa reinforcing again that the negative control was reasonable (Fig.2c). The culture of S. aureus a Gram-positive, round-shaped bacterium that is a member of the Firmicutes, and it is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin was also not affected by the methanolic extract of *Nyctanthes arbor-tristis L.* at the concentrations 9 µg (1), 18 µg (2) and 25 µg (4) (Fig.3). Here too DMSO control at 10% did not affect the growth of P. aeruginosa reinforcing again that the negative control was reasonable (Fig.2d).

Since the methanolic extract of the flowers of Nyctanthes arbor-tristis L. at the concentrations 9 µg (1), 18 µg (2) and 25 µg (4) did not have much inhibition on the growth of the four bacterial cultures namely Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli an attempt was made to see if the methanolic extract of the flowers of *Nyctanthes arbor-tristis L.* might have any synergistic effect along with conventional antibiotics such that it might enhance the overall efficacy of the antibiotic. The culture of Pseudomonas aeruginosa was used for the synergism study. Commonly used antibiotics to control bacterial infections like Cefotaxime 30 µg (CTX- 30), Cefixime 0.5 µg (CFM- 0.5), Cefpodoxime 10 µg (CPD - 10) and Tetracyclin 30 µg (TE-30) were soaked in autoclaved filter paper discs and added to the plate of Pseudomonas aeruginosa culture. This plate served as a positive control plate (Fig. 3a). For analyzing if the synergistic effect is there for the methanolic extract of the flowers of Nyctanthes arbor-tristis L. a higher concentration of 52 µg was used in the medium to culture Pseudomonas aeruginosa (Fig. 3b). Commonly used antibiotics to control bacterial infections like Cefotaxime 30 µg (CTX- 30), Cefixime 0.5 µg (CFM- 0.5), Cefpodoxime 10 µg (CPD - 10) and Tetracyclin 30 µg (TE-30) were soaked in autoclaved filter paper discs and added to the plate of Pseudomonas aeruginosa culture in the methanolic extract of the flowers of *Nyctanthes arbor-tristis L.*(Fig. 3).

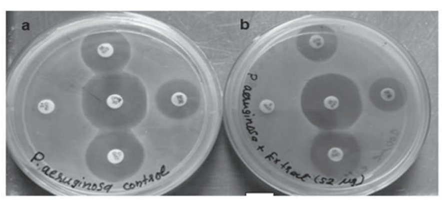

Fig.3 Synergestic effect of methanolic extract of *Nyctanthes arbor-tristis L.* flowers on Pseudomonas aeruginosa at 52 µg along with different antibiotics in discs (Cefotaxime 30 µg (CTX- 30), Cefixime 0.5 µg (CFM- 0.5), Cefpodoxime 10 µg (CPD - 10) and Tetracyclin 30 µg (TE-30) (b) along with control plate without any methanolic extract of *Nyctanthes arbor-tristis L.* flowers but with antibiotic disc alone Cefotaxime 30 µg (CTX- 30), Cefixime 0.5 µg (CFM-0.5),Cefpodoxime 10 µg (CPD - 10) and Tetracyclin 30 µg (TE-30) (a)

The absence of any difference between the zone of inhibition in the plate with antibiotic disc alone (control plate of Pseudomonas aeruginosa) (Fig. 3) and the plate of Pseudomonas aeruginosa cultured in a medium with methanolic extract of the flowers of *Nyctanthes arbor-tristis L.* was the observation.

Essential oils are commercially important as well as in the point of view of medicinal value, and their utility in aromatherapy is widely accepted. The essential oil from the flowers of Nyctanthes arbor-tristis L.has also been shown to have immense potential in many systems of medicine and aromatherapy (Agrawal and Pal 2013). Since the essential oil of flowers of Nyctanthes arbor-tristis L. also had immense commercial value, we tried to tap it by Clevenger mediated hydrodistillation. Although cold press methods were frequently used in the extraction of volatile oils that were heat-labile, we adopted the Clevenger method to try and see if the aromatic element of the flowers of *Nyctanthes arbor-tristis L.* could indeed be isolated and harvested by hydrodistillation using Clevenger (Siriwardena and Arambewela 2014). Although Clevenger mediated hydrodistillation method was attempted to isolate and extract the essential oil from the flowers of Nyctanthes arbor-tristis L., the water extract was the only output. The fragrance of the aqueous extract was also quite different from that of fresh flowers of *Nyctanthes arbor-tristis L.* Hence it was pretty clear to us that alternative methods of extraction like cold press method and advanced methods of detection using Mass spectrometer using Headspace attachment might be required to identify the volatile constituent in the flowers of *Nyctanthes arbor-tristis L.* that gave them its unique fragrance (Naik et al.2016).

Nevertheless, we could understand and study in details the nuances of extraction of essential oils and volatile constituents from flowers of Nyctanthes arbor-tristis L. Even though the fragrance of aqueous extract of *Nyctanthes arbor-tristis L.* flowers was different from fresh flowers, it seemed to have a soothing or even sedative effect when inhaled. This was proof to the fact that although the heat of the hydrodistillation process-induced specific changes in the chemical composition of the volatile ingredients, it still retained some of its therapeutic potentials. Aromatherapy is usually associated with inducing calmness or soothing effect, and hence this aqueous extract from the flowers of *Nyctanthes arbor-tristis L.* derived from Clevenger process of hydrodistillation could still be of immense use. The thermolabile constituents of essential oils have indeed been shown to have potent activity than their parent constituents from which they were derived although in a different context of pest management (Tripathi et al.2009).

Even though the antibacterial and cytotoxic activity of flowers of Nyctanthes arbor-tristis L. has been studied in a variety of bacteria both gram-positive and gram-negative, the extracts were in Chloroform, Ethyl acetate or Petroleum Ether. Also, ethanolic extracts were used to study cytotoxicity, whereas Chloroform or Ethyl acetate fractions were used to study antibacterial activity by calculating the zone of inhibition (Khatune et al. 2001). This study was not comprehensive and did not include methanolic extract. Also, this study did not include Klebsiella pneumoniae a gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacteriumas a microorganism. Our study is thus exploring this aspect. In the previous study, petroleum ether fraction showed no antibacterial activity, but mild to moderate activity was reported in ethanol, chloroform or ethyl acetate fractions. But an important point worth mentioning here is that the concentrations of crude fractions used in this study were very high (200μg and 400μg). Such high concentrations are not achievable in a therapeutic setting. Hence our study focussed on the methanolic fraction from flowers of Nyctanthes arbor-tristis L. as methanol is the most polar of solvents and since the aqueous extract from Clevenger showed fragrance, we expected maximum yield of essential oil constituents in methanolic fraction. It was reported in another study that most phytochemical constituents and polar components, in particular, would be eluted to the maximum in methanolic fractions (Sah and Verma,2012).

The high therapeutic potential of Nyctanthes arbor-tristis L. seems to be specified regarding the leaves and bark of the plant (Manisha et al.2009). An enormous amount of studies have focussed on the therapeutic potential of extracts from the leaves and bark of Nyctanthes arbor-tristis L. while only a few reports were available on that of flowers of *Nyctanthes arbor-tristis L.*(Rathee et al. 2007). Cytotoxicity, antiproliferative and antioxidant study of different solvent extracts of Nyctanthes arbor-tristis L. flowers was reported (Khanapur et al.2014). The results from this particular study also showed that methanolic extract of the flowers of Nyctanthes arbor-tristis L. was more potent when compared to other solvents such as petroleum ether.

The present study was not very convincing regarding the antibacterial activity of methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* on account of many reasons. The first being that the crude fraction in methanol contains many pigments which include many carotenoids, flavonoids and antioxidants. Antibacterial activity of many extracts and antibiotics is due to their ability to induce reactive oxygen species induced damage in microbes. The presence of antioxidants in the methanolic fraction might mitigate the reactive oxygen species generation, and this might have blunted the actual effect of the methanolic extract (Singh and Vyas2018).

Another reason for the reduced antimicrobial activity of the methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* might be because of interference from the pigment Nyctanthin. The pigment Nyctanthin, which was mainly present in the petiole of the flower of *Nyctanthes arbor-tristis L.*, gets diffused in the medium of methanol. The pigment reduced the visibility of bacterial lawn culture, especially in wells and around wells on bacterial agar plates into which the methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* was added. The visibility around the wells was low, owing to the intense colour of the pigment Nyctanthin. The low visibility region which appeared very dark was restricted to the periphery of the wells in low concentration (9μg) of methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* With the increase in concentration to 18 μg , the increased pigmentation was diffusing more outward of the well to a length of approximately. At 25µg of methanolic fraction from flowers of *Nyctanthes arbor-tristis L.*, the low visibility region around the well was augmented to a perimeter of 1cm.

A halo of mild pigmentation of 2 cm length could be seen around the wells added with a methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* at the concentration of 9µg . This halo extended to a length of 2.5 cm around the wells added with a methanolic fraction from flowers of Nyctanthes arbor-tristis L. at a concentration of 18 µg . The region or halo further extended to a length of 3 cm around the wells added with a methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* at a concentration of 25µg .

The dark region, along with the halo region formed by the diffusion of the dye Nyctanthin, prevented visibility of zone of inhibition if any around the wells. Hence the diffusion of the pigment Nyctanthin was the major impediment in the positive outcome of the present study. Hence an alternate approach was tried. It has been previously reported that certain natural products have a synergistic effects along with regular antibiotics to inhibit the growth of microbes thereby helping us reduce the dose of antibiotics and reducing the development of drug-resistant strains of microbes (Kumar et al.2013).

The synergistic effect of methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* along with conventional antibiotics like Cefotaxime, Cefixime, Cefpodoxime and Tetracyclin has not been reported earlier and seemed a novel approach. Hence to see if methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* enhanced the efficacy of conventional antibiotics, the antibiotics were absorbed on Whatman III filter paper discs and placed on a petri dish containing a culture of Pseudomonas aeruginosa (Fig. 3b) at a concentration of Cefotaxime 30 µg (CTX- 30), Cefixime 30 µg (CFM 30), Cefixime 0.5 µg (CFM- 0.5), Cefpodoxime 10 µg (CPD - 10) and Tetracyclin 30 µg (TE-30) respectively. This experiment served as a positive control to show that these antibiotics indeed inhibited the growth of Pseudomonas aeruginosa and produced a zone of inhibition that was markedly visible (Fig.3a). Cefixime 30 µg produced the maximum zone of inhibition (3cm), followed by Cefotaxime 30 µg which produced a zone of inhibition of 2 cm and Cefpodoxime at a concentration of 10 ?g produced a zone of inhibition of 2.2 cm. Tetracyclin at 30 µg produced a zone of inhibition of 1.5 cm. The least activity in terms of zone of inhibition was exhibited in Cefixime at a concentration of 0.5 µg with no discernible zone of inhibition (Fig.3a).

For analyzing the synergistic effect of methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* along with conventional antibiotics like Cefotaxime, Cefixime, Cefpodoxime and Tetracyclin, methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* at a concentration of 52 µg was added to the plate along with a liquid culture of Pseudomonas aeruginosa and spread. Discs containing conventional antibiotics like Cefotaxime 30 µg (CTX- 30), Cefixime 30 ?g (CFM 30), Cefixime 0.5 µg (CFM- 0.5), Cefpodoxime 10 µg (CPD - 10) and Tetracyclin 30 µg (TE-30) respectively were placed in identical locations similar to that of the previous plate(Fig.3 a &b).

Cefixime 30 µg produced the maximum zone of inhibition (3cm), followed by Cefotaxime 30 µg which produced a zone of inhibition of 2 cm and Cefpodoxime at a concentration of 10 µg produced a zone of inhibition of 2.2 cm. Tetracyclin at 30 ?g produced a zone of inhibition of 1.5 cm. The least activity in terms of zone of inhibition was exhibited in Cefixime at a concentration of 0.5 µg with no discernible zone of inhibition (Fig. 3 b). The addition of methanolic fraction from flowers of Nyctanthes arbor-tristis L. had a little inhibitory effect on the growth of Pseudomonas aeruginosa in combination with the antibiotics at the given concentrations. This was assumed based on the fact that no discernible difference in a zone of inhibition was observed between the plate of Pseudomonas aeruginosa culture without plant extract and the plate of Pseudomonas aeruginosa culture with the addition of methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* at a concentration of 52 µg (Fig.3 a and 3b).The absence of synergism between methanolic fraction from flowers of Nyctanthes arbor-tristis L. and conventional antibiotics could be possibly attributed to their different modes of action. It is not a complete surprise because the inhibitory effect of methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* alone at concentrations 9 µg, 18 µg and 25 µg was not discernible in terms of zone of inhibition in different strains of microbes like Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus.

Despite the negative results for the antimicrobial activity of methanolic fraction from flowers of *Nyctanthes arbor-tristis L.* in the present study, the importance of the pharmacological value of this plant can never be sidelined (Rani et al.2012). The cytotoxic activity of other parts of the plant along with several reports on its antiproliferative activity concerning cancer cell lines points to the immense potential of this plant as an anticancer agent (Jain and Pandey2016). The application of this plant in rejuvenation therapy is another area that has immense potential (Santosh and Manojkumar 2016). The separation of different fractions in the crude methanolic extract of the flowers of *Nyctanthes arbor-tristis L.* by column chromatography is the way forward. Such an approach might yield better results in terms of antimicrobial activity since active fractions, and active ingredients alone have been shown to have greater potency in terms of antimicrobial activity compared to that of their crude parent fractions (Kaur and Kaushal 2020).

Conclusion

The active fractions from the crude methanolic extract from the flowers of *Nyctanthes arbor-tristis L.* exhibited marginal antimicrobial activity. Separation of Nyctanthin pigment from the active fractions is indeed inevitable for creating a zone of inhibition. The synergistic inhibitory effect between conventional antibiotics and active fractions derived from the crude methanolic extract from the flowers of *Nyctanthes arbor-tristis L.* displayed a diffused zone of inhibition and hence is thought to have marginal antibacterial efficacy. The study demonstrated that the cold-press method for isolation of essential oil from the flowers of *Nyctanthes arbor-tristis L.* might be more effective an extraction procedure. Crude methanolic extract from the flowers of *Nyctanthes arbor-tristis L.* must be subjected to fractionation by column chromatography for proving its potency.

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