Screening and Evaluation of Phytochemical Constituents, Antimicrobial, Antioxidant and Anti-Arthritic Activities of Methanolic Extract of *Nyctanthes arbortristis L.* Leaves

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Abstract

The objective of the current research was to determine the antioxidant, antimicrobial and anti-arthritic activities of the methanolic extract of Nyctanthes arbortristis leaves (MLEN). In the present study, the phytochemical constituents, total phenolic content and reducing power of MLEN were analysed and the antimicrobial activity of MLEN against three clinical pathogens: Staphylococcus aureus (MTCC 9542), Fusobacterium nucleatum (MTCC 25586) and Propionibacterium acnes (MTCC 1951) was studied using the agar-well diffusion method. We evaluated the antioxidant characteristics and anti-arthritis activities using the DPPH assay and in vitro inhibition of egg albumin denaturation. MLEN showed the presence of phenols, tannins, cardiac glycosides, alkaloids and flavonoids, with a total phenolic content equivalent to 450 µg/ml of gallic acid and a maximum reducing power (0.88) at 80 µg/ml.

The leaf extract showed 28.28 mm/0.1 ml inhibition with S. aureus, 25.75 mm/0.1 ml with F. nucleatum and 13.18 mm/0.1 ml with P. acnes. MLEN showed significant antioxidant activity (IC50 59.7 µg/ml). In vitro anti-arthritic activity, achieved through the inhibition of protein denaturation, yielded promising results with an IC50 of 109.3458 µg/ml. Outcomes of the investigation revealed that phenols, tannins, cardiac glycosides, alkaloids and flavonoids may be the primary metabolites in MLEN responsible for significant antimicrobial, antioxidant and anti-arthritic activities, paving the way for further investigation of biologically active principles of pharmacological significance.

Keywords: *Nyctanthes arbortristis*, phytochemical screening, antimicrobial activity and antioxidant potential.

Introduction

Nowadays, pharmaceutical research on herbal medicine is gaining more attention due to its therapeutic use as an alternative to conventional medicine, as plants are a rich source of relatively harmless, potentially beneficial chemical compounds that can be used as drugs to treat various human ailments[1,4,14]. *Nyctanthes arbortristis* is one of the most popular plants in Ayurveda and herbal medicine with extensively wealthy phytochemical constituents, some of which are still to be explored.

N. arbortristis (night-flowering jasmine, coral jasmine and Parijat), which belongs to Nyctaginaceae, is a large shrub having a broad spectrum of pharmacological properties and is extensively cultivated in tropical and subtropical areas from India to Nepal, from the outer Himalayas and Jammu and Kashmir to east of Bengal, Tripura and Assam, up to the central part of the extended Godavari in the south[5,8] and is commonly found in red as well as black soils with a 5.6 to 7.5 pH[30]. Ayurveda, Siddha and Unani medicine systems used *N. arbortristis* as a laxative, digestive aid, diuretic, expectorant, antivenom and mild bitter tonic[2,10,34,41].

The phytochemical evaluation of *N. arbortristis* is gaining significance due to the increasing interest in natural products as an alternative source of medicine. It makes a significant contribution in expanding the knowledge of the chemical constituents of *N. arbortristis and* provides a scientific basis for its traditional uses. The exploration of plant-based bioactive secondary metabolites, their isolation, purification, characterisation and identification of specific bioactive compounds, will play a pivotal role in developing novel drugs or therapeutic agents. Several phytochemical constituents, including carbohydrates, steroids, flavonoids, iridoid glycosides, terpenes and alkaloids, available in different parts of *N. arbortristis*, exhibited varying pharmacological activities[12,45].

Quinolinolids, 7-(alpha-anilino-p-nitro benzyl)-8-quinolinol, protoberberines and aporphines reported in the leaves of *N. arbortristis* exhibited anti-inflammatory properties[19]. Methanolic stem bark extract of *N. arbortristis* exhibits anti-inflammatory properties, as was reported in Wistar albino rats (assessed by carrageenan-induced rat paw oedema)[18]. Similarly, seed extracts demonstrated potential antioxidants[26,31], antibacterial properties[3,6,43]. Polyphenol extract of *N. arbortristis* leaves has been reported to contain gallic acid, chlorogenic acid, protocatechuic acid and caffeic acid, exhibiting effective free radical scavenging activity[24]. This current research aimed to demonstrate the antimicrobial and antioxidant activities of bioactive phytochemical constituents present in the methanolic leaf extract of *N. arbortristis* to identify lead molecules of pharmacological significance.

Material and Methods

Collection of plant material and authentication: The collection of *N. arbortristis* leaves was carried out in distinct areas of the Eluru district, Andhra Pradesh, India. Prof. S. M. Khasim, Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur 522510, Andhra Pradesh, India, authenticated the plant's taxonomic identity.

Extraction of the plant material: Fresh leaves from *the N. arbortristis* plant were washed, dried at room temperature and then crushed into a powder using a mechanical grinder. The leaf extract was prepared by mixing 258 g of leaf powder with 3100 mL of methanol. Methanolic extract (MLEN) was prepared at 35-40°C in the Soxhlet apparatus and concentrated to 1 mg/ml (W/V) under reduced pressure using a rotary evaporator and stored at 4°C until use[4].

Chemicals and Clinical Pathogens: Clinical pathogens *(Staphylococcus aureus*-MTCC 9542, *Fusobacterium nucleatum*-MTCC 25586 and *Propionibacterium acnes*-MTCC 1951), Mayer's reagent, cell culture medium NAM, DPPH, 10 ml serological pipettes (TORSON), T25 flask (#12556009, Biolite-Thermo) and 50 ml centrifuge tubes (#54 043 TORSON) were used. Methanol was purchased from Sigma-Aldrich Co., USA.

Equipment: Soxhlet apparatus (Borosil), Rotary Evaporator (Heidolph, Germany), Centrifuge (Remi: R-80C), Hot Air Oven (Healforce, China), Incubators (Healforce, China), Pipettes: 2-10 µL and 10-100 µL, UV-Spectrophotometer were used.

Phytochemical constituents: A phytochemical screening of the extract was carried out using various tests to detect the compounds. Carbohydrates by Molisch's test, alkaloids by Mayer's test, glycosides by the foam test for saponin glycosides and Bontrager's test for both cardiac glycosides and anthraquinon glycosides. Flavonoids were tested with the lead acetate test and tannins were identified using the ferric chloride test. Terpenoids were assessed using the salkowski test, while fats and oils were screened with the saponification test. Coumarins were detected using the fluorescence test and proteins were identified using biuret's test[4].

Quantitative Analysis of Total Phenolic Content [TPC]: Total phenolic content of extracts was determined as described. One ml of extract (1 µg/ml) was added to 1 ml of Folin-Ciocalteu's reagent and saturated with Na_2CO_3 for 3 minutes. The mixture was diluted to a final volume of 10 ml and incubated for an hour in the dark. Absorbance was measured at 725 nm. Gallic acid was taken as the standard.

Estimation of Reducing Power: Different concentrations of the extract were added to 1 mL of distilled water and 1 mL of 0.2 M phosphate buffer, along with 1% ($K_3[Fe(CN)_6]$). The mixture was incubated for 20 min at 50°C. To this, 1 mL of 100 mg/mL trichloroacetic acid was added and the mixture was centrifuged for 10 minutes at 8000 × g. Distilled water and $FeCl_3$ were added to the upper layer of solution. At a wavelength of 700 nm, absorbance was measured. As a standard reference, ascorbic acid was used.

Antimicrobial activity: The antimicrobial activity of the plant extract has been studied using the agar well diffusion method, with methanol as the control. Three bacterial strains, namely *Staphylococcus aureus (MTCC* 9542*), Fusobacterium nucleatum (MTCC* 25586) and *Propionibacterium* acnes (MTCC 1951), were obtained from MTCC, Chhattisgarh and cultured in nutrient agar medium. The streaking technique was used to inoculate the bacterial culture with a concentration of 10^8 CFU. 100 µL and 200 µL of MLEN and 100 µL of methanol, were separately poured into wells with an 8 mm diameter and incubated for 24 to 48 hours at 37°C. The diameter of inhibitory zones surrounding each well was measured in mm following incubation. Methanol served as a negative control. Streptomycin served as a positive control. Zones of inhibition were measured by measuring their diameters.

DPPH free radical scavenging assay: DPPH was used to assess MLEN's scavenging capacity for free radicals. 3 mL of MLEN was prepared in different concentrations (20, 40, 60, 80, 100 µg/mL). 1 mL of 0.004% methanolic DPPH was added to each concentration and placed in the dark for 30 minutes. Ascorbic acid was used as a standard and the absorbance was measured at 517 nm. The % inhibition was calculated using the formula:

% inhibition = [[absorbance of control – Absorbance of sample] / Absorbance of control] × 100.

***In vitro* anti-arthritic activity:** The *in vitro* anti-arthritic property was determined by the inhibition of egg albumen denaturation. 5-ml reaction mixture is composed of 0.2 ml egg albumin, 2.8 ml phosphate-buffered saline and 2 ml MLEN and is incubated at 37°C for 15 minutes, followed by incubation at 70°C for 5 minutes. The effective dose was determined using extracts at varying concentrations (20, 40, 60, 80, 100 µg/ml), with 5 ml of double-distilled water as the control. Absorbance was measured at 660 nm at room temperature. Various doses of diclofenac sodium (20, 40, 60, 80 and 100 µg/ml) were taken as the reference medication. The formula for calculating the % inhibition of protein denaturation is:

% of inhibition = [Vc-Vt/Vc] x 100

where Vc is absorbance of control and Vt is Absorbance of test sample

Results and Discussion

Phytochemical screening: Research on medicinal plants for their chemical constituents has become increasingly important due to their therapeutic value and potential. As part of the current investigation, a crude methanolic extract

of *N. arbortristis* leaves has been analysed for its chemical constituents. As reported earlier[7,16,22,33,37,38,45,48], MLEN also contained alkaloids, flavonoids, glycosides, terpenoids, tannins and saponins (Table 1). In the essential oil of *N. arbortristis*, compounds such as geranylgeraniol, cis-9-tricosene, benzyl salicylate, n-pentacosane, phytone, nonadecane and methyl stearate were found to be present while the anthraquinone compound was absent[42]. The metal analysis studies on *N. arbortristis* revealed the presence of heavy metals at permissible levels, indicating that the plant was safe for use in herbal drug formulations[46].

Quantitative Analysis of Total Phenolic Content [TPC]: Phenols present in the plant extract reacted with phosphomolybdenum and produced a blue colour. The plant extract exhibits an absorbance of 1.47 at 725 nm, equivalent to 450 µg/ml of gallic acid, as determined by the Folin and Ciocalteu method.

Estimation of reducing power: When estimating the reducing power of plant extracts, the absorbance values at 700 nm increased as the concentration of the plant extract increased. The maximum absorbance was recorded at 0.88 at a concentration of 80 µg/ml. It coincided with the concentration of the highest antioxidant activity, which is 80 µg/ml, indicating the optimum concentration of plant extract for antioxidant activity (Figure 1).

Antimicrobial activity: Three bacterial strains were employed to assess the plant extract's antibacterial properties using the agar well diffusion method, namely *S. aureus* (MTCC 9542), *F. nucleatum* (MTCC 25586) and *P. acnes* (MTCC 1951). The zones of inhibition at 100 µL concentrations for these bacteria are 4.47 ± 0.40 mm, 4.09 ± 0.40 mm and 2.11 ± 0.40 mm respectively. At a 200 µL concentration, the zones of inhibition are 5.66±0.40 mm, 4.41±0.40 mm and 3.65±0.40 mm respectively. The results indicated that plant extract is most effective on *S. aureus* (MTCC 9542), moderately effective on *F. nucleatum* (MTCC 25586) and least effective on *P. acnes* (MTCC 1951) (Fig. 2 and Table 2).

The aqueous extracts of dried leaves demonstrated anti-malarial activity in an experimental study on patients suffering from malaria in Worli, Mumbai[13]. Ethanolic, methanolic and chloroform leaf extracts showed a marked antibacterial activity against different pathogenic bacteria including *S. aureus, P. acnes, Pseudomonas aeruginosa and Salmonella typhi*[10,32,40]. With an inhibition zone diameter of 23.8 as well as 26.3 mm at 1000 µg/ml in opposition to *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* respectively, essential oil eugenol and its derivatives from dried leaf extracts of *N. arbortristis* had the most potent antibacterial activity[20]. Ethyl acetate and dichloromethane extracts of *N. arbortristis* exhibited good antioxidant activity against *B. subtilis, B. cereus and Pseudomonas*[15]. *N. arbortristis* plants have excellent antimicrobial activity[29]. According to the results above, the antimicrobial activity of each bacterial species increases as the concentration of the plant extract rises. The maximum activity was observed with *S. aureus* while it showed the least effect on *P. acnes* at both concentrations. Inhibition of *F. nucleatum* is moderate compared to the other two species.

Antioxidant activity: A DPPH assay was used to quantify MLEN's capacity to scavenge free radicals. The inhibition percentage gradually increased with an increase in the concentration of the plant extract from 20 to 80 µg/ml and then declined at 100 µg/ml. Maximum % of inhibition was observed at 80 µg/ml with an IC50 value of 59.7 µg/ml, indicating potent antioxidant activity of plant extract (Fig. 3). This result was supported by a study by Michael et al[25] who displayed an IC50 value of 57.93 µg/ml with MLEN. High antioxidant activity has been proved with methanol[39] and ethyl acetate extracts of root[9], stem and flower of *N. arbortristis*, with excellent scavenging action of aqueous extract of leaf[17].

In rats induced with diabetes by streptozotocin, Rathod et al[36] demonstrated the ability to scavenge free radicals. Hydroethanolic leaf extract of *N. arborists* showed good free radical scavenging activity on the liver of a Freund's adjuvant-induced arthritis model[23]. Devasree et al[11] successfully noted the impact of *N. arbortristis* leaf aqueous extract on *the* immunological response of *Oreochromis mosambicus*. The ethanolic leaf extract of *N. arbortristis* showed high antioxidant property in high-fat streptozotocin diet-induced diabetic rats[27]. Significant antioxidant activity of ethyl acetate extract from the leaf was noticed because of its scavenging ability of ROS, DNA strand breaks and lipid peroxidation[35] and antioxidant activity in different solvent extracts of flowers[21]. The antioxidant activity was higher in the calyx than in the petals of the aqueous flower [47]. Extracts of N. *arbortristis* showed potential antioxidant activity[44]. Comparative analysis of the antioxidant activity of a plant's roots, flowers, leaves and seeds with successive extracts, methanolic and aqueous extracts showed more antioxidant capacity than petroleum ether and chloroform extracts. The free radical scavenging activity is attributed to various bioactive compounds such as polyphenols, present in the plant parts[17].

Anti-arthritic activity: Comparing MLEN to the standard medication diclofenac, the current investigation showed a significant decrease in protein denaturation. Protein denaturation inhibition % increased with concentration, with an IC50 of 109.3458 µg/ml. At 60 µg/ml, 80 µg/ml and 100 µg/ml, the plant extract's inhibition percentage is higher than that of diclofenac (Fig. 4). One of the main concerns of inflammation in arthritis is protein denaturation. In some arthritic diseases, protein denaturation, along with the action of proteases, may lead to the production of autoantigens which are characteristic of certain arthritic conditions[4]. Leukocyte proteinase causes tissue damage during inflammatory reactions and by inhibiting proteinase activity, protection is provided against inflammation[36].

Conclusion

The availability of phenols, carbohydrates, flavonoids, alkaloids, glycosides, terpenoids and saponins was revealed through the screening of the phytochemical elements of *Nyctanthes arbor-tristis*. *Staphylococcus aureus* is the strain most responsive to the concentration-dependent inhibition of bacterial growth via methanolic leaf extract from *N. arbor-tristis*. It supports the possible application of *N. arbor-tristis* as a natural antimicrobial agent. Future investigations will be needed to optimise concentration and to explore its mechanism of action.

Table 1
Phytochemical constituents of MLEN

Phytochemicals	MLEN
Alkaloids	+
Flavonoids	+
Glycosides	+
Terpenoids	+
Tannins	+
Saponins	+
Anthraquinones	-

Table 2
Antimicrobial Activity of MLEN [Zone of Inhibition in mm]

Bacterial Strain	Zone of Inhibition [100µL]	Zone of Inhibition [200µL]	Circumference of inhibition mm/0.1ml
S. aureus	4.47± 0.40	5.66±0.40	28.28
F. nucleatum	4.09± 0.40	4.41±0.40	25.75
P. acnes	2.11 ±0.40	3.65 ± 0.40	13.18

Values are represented in Mean ±SD (n=3)

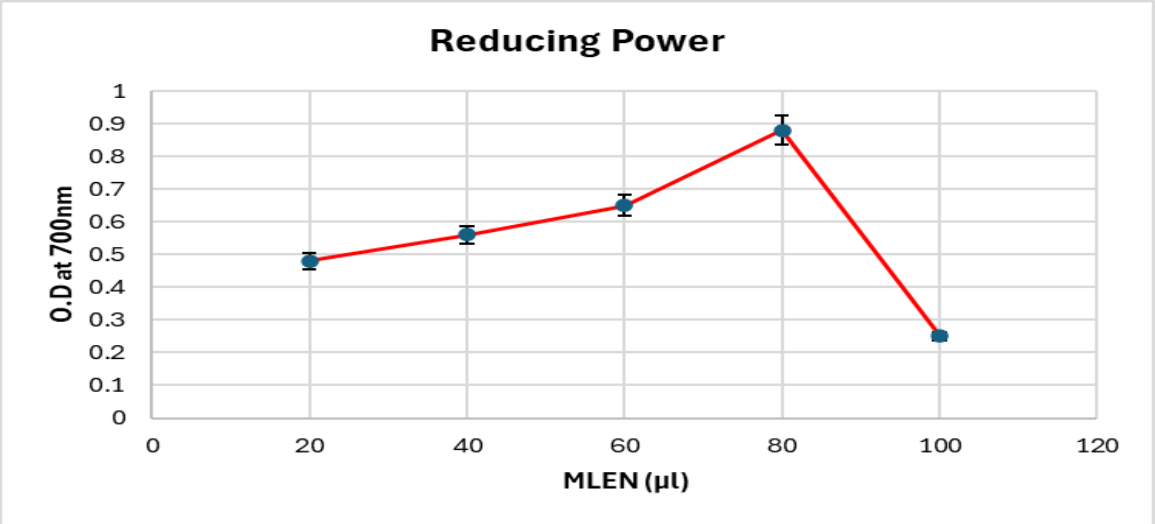

Fig. 1: Estimation of reducing power of MLEN

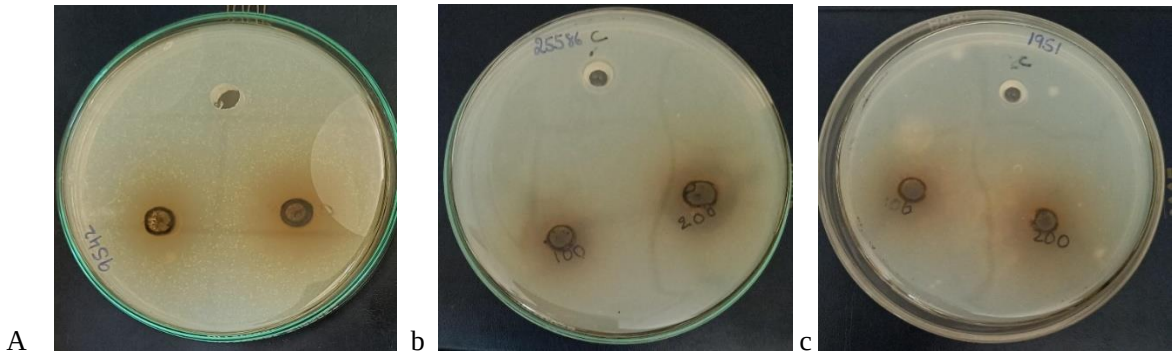

Fig. 2: Antimicrobial Activity of MLEN
[a] *Staphylococcus aureus* [b] *Fusobacterium nucleatum* and [c] *Propionibacterium acnes*

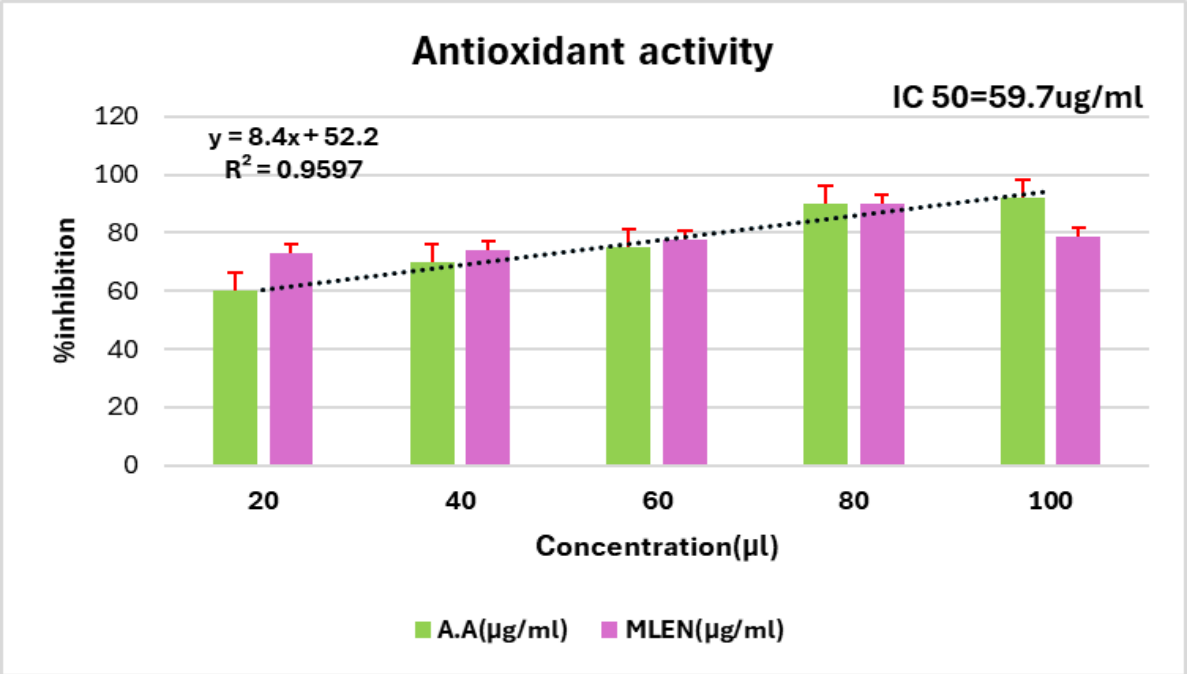

Fig. 3: Antioxidant activities of MLEN in comparison with Ascorbic acid

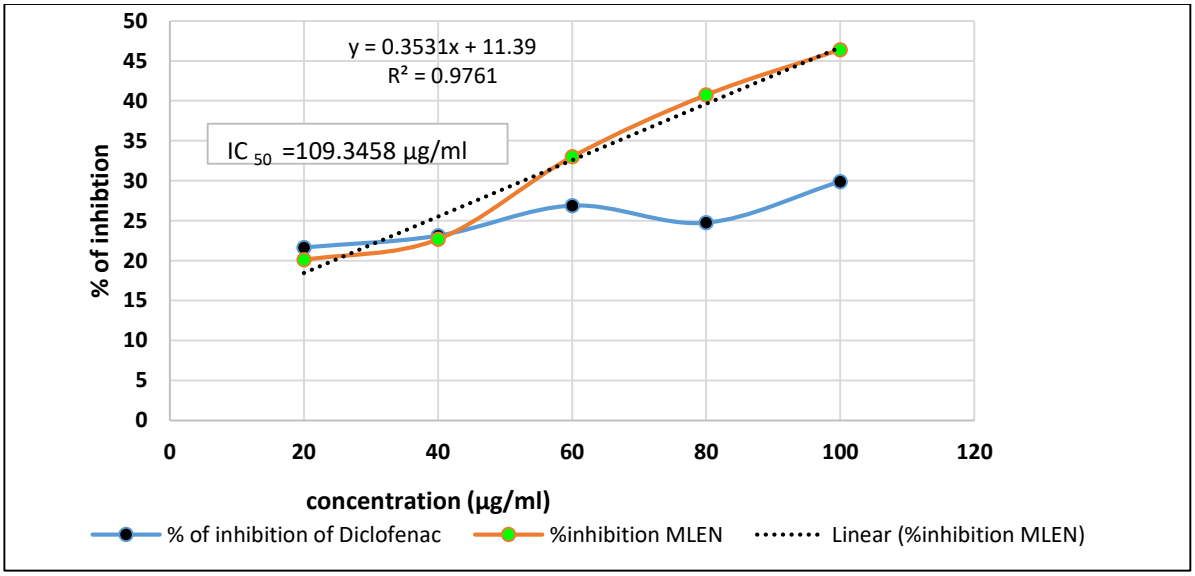

Fig. 4: Antiarthritic activities of MLEN in comparison with Diclofenac

While studying the antioxidant activity of plant extract, the plant extract demonstrates an initial increase in inhibition, peaking at 90.09% at 80 µg/ml, followed by a decline to 78.59% at 100 µg/ml, indicating a possible optimal concentration for effectiveness. While estimating the declining capacity of the plant extract, absorbance increases with plant extract concentration up to 80 µg/ml, indicating higher antioxidant or reactive activity. However, a drop in absorbance at 100 µg/ml suggests a non-linear response, possibly due to saturation or inhibitory effects.

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