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Assessment of Antioxidant, Antimicrobial, Anti-inflammatory and Immunomodulatory Properties of *Myrica esculenta*

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ABSTRACT

The central Himalayan state of Uttarakhand is endowed with an abundance of different medicinal and aromatic plants (MAPs), which have long been utilized in Siddha, Ayurveda and regional folk medicine. This study investigated whether the ethanolic leaf extract of *Myrica esculenta* modulated the production of nitric oxide (NO). This ethanolic leaf extract was prepared and determined for the existence of secondary metabolites using high-performance thin-layer chromatography (HPTLC) and also by estimating the total phenolic and flavonoid content along with antimicrobial activity. Additionally, it was examined how they influenced the J774 macrophage cell line's generation of NO and TNF-alpha pro-inflammatory cytokine production when stimulated by lipopolysaccharide (LPS), which was measured using enzyme-linked immunosorbent assay (ELISA). The results showed that the ethanolic leaf extract contained terpenoids, phenolics and glycosides, as confirmed by HPTLC, and it also had a higher content of phenolics and flavonoids. In addition, leaf extracts showed significant antimicrobial, anti-inflammatory (protein denaturation and membrane stabilization) and cytotoxicity activity at higher concentrations. In a cell culture assay using the J774 cell line after 24-48 h of treatment with ethanolic leaf extract, the cells showed a significant decrease in NO production along with TNF alpha and IFN gamma for ethanolic leaf extract at a concentration of 1000 ìg/ml. Acetaminophen was the gold standard for suppressing NO generation at 1 µg/ml following 24 and 48 h (nearly 95% inhibition) in contrast to the ethanolic leaf extract of *M. esculenta*. These results demonstrated that extracts containing anti-inflammatory and macrophage-inhibiting compounds, may offer a different approach to treating various inflammatory illnesses.

Key words: *Myrica esculenta*, antioxidant activity, cell line, nitric oxide, tumor necrosis factor

INTRODUCTION

The ancient Indian medical system termed ayurveda lays a strong focus on using medicinal herbs to cure an assortment of illnesses, prevent disease and promote wellness. One of the most advantageous places for acquiring ayurvedic medicinal plants is the Himalayan area, which is frequently referred to as the "abode of herbs". Himalayan medicinal plants' curative effects and great diversity make them essential to both conventional and futuristic health care systems. Several plant genera that have considerable medicinal potential may be identified around the mountainous area that has been acknowledged as a hotspot for biodiversity. Several of these species of plants have been utilised in Tibetan, Ayurvedic, Unani and folk medicine (Bhat *et al.*, 2021). These plants have naturally occurred bioactive substances ranging from alkaloids and flavonoids to tannins, as well as essential oils that aid the medical management of several illnesses, such as infections, diabetes, inflammation, respiratory diseases, digestive difficulties and cardiovascular concerns (Chaudhary and Singh, 2023). These medicinal plants constitute the main source of easily accessible and economically viable remedies among impoverished and isolated Himalayan populations (Bisht *et al.*, 2019). Along with providing opportunities for the creation of novel medications and herbal formulations, they additionally assist pharmacological research and the nutraceutical sectors globally (Bhandari *et al.*, 2020). Thus, Himalayan medicinal plants serve as a basis for sustainable healthcare and economic growth in addition to preserving indigenous knowledge and cultural legacy (Tungmunnithum *et al.*, 2018).

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Myrica esculenta, commonly known as bayberry or Kaphal, is a dioecious evergreen shrub or small tree belonging to the genus *Myrica* in the Myricaceae family. The plant is known for its fruit and other uses, and it has a local name "Kaphal" which shows its relation to the culture, tradition and medicine of the particular region, which is historically used against multiple ailments like gastro-intestinal disorders, respiratory ailments and skin diseases (Kabra *et al.*, 2019). The importance and versatility of *M. esculenta* regional health practices are characterized by the regional differences in its use (Sood and Shri, 2018). In Bhutan, the herb has healing properties of wound and skin disorders through topical treatments and poultices. These local customs highlight the plant's many uses and incorporation into many traditional medical systems (Mishra and Pandey, 2023). Thus, the study determined that *M. esculenta* is a shining illustration of their traditional knowledge and medicinal properties, influencing at various cultures and geographical areas (Alcalde *et al.*, 2019). The objective of this study was related to the ethanolic extract of *M. esculenta* leaves and analyzing its antioxidant potential and analyzing the nitric oxide estimation along with its cytotoxicity in cell culture.

MATERIALS AND METHODS

The newly harvested leaves of *Myrica esculenta* were harvested in April 2025 from the Pauri Garhwal region in Uttarakhand, India. The collected plant fragments were recognised by the Forest Research Institute in Dehradun, India. The fresh leaves from the plant were first cleaned with water to get rid of any dirt that could have adhered to them. After that, they were cut into little pieces and left in a shade region for seven consecutive days. The entire amount was dried well, then ground into a coarse powder and stored in a sealed container for later use.

An aggregate of 500 g of plant material was submerged in 95% ethanol for a whole day at a concentration of 1:10. The rotary evaporator operated under 60°C, at 80 rpm. Then the ethanolic solvent was used to filter and concentrate the filtrate solution. The crude extract was kept in a fridge at 2-4°C.

After extracting 0.5 g of the *M. esculenta* (leaves) in a total of 10 ml of ethanol, it was spun in a centrifuge for 5 min at a speed of 3000 rpm. The supernatant was then applied for HPTLC examination of terpenoids, phenolics and glycosides. A LINOMAT 5 applicator (CAMAG) with a 7 mm band length and an 8 mm separation from the Y-axis was used for the sample. A 20 × 10 cm silica gel $60F_{254}$ plate was used for the chromatographic separation, and the solvent front was kept at 80 mm (Mondal *et al.*, 2017). The phase that moved around consisted of n-hexane and ethyl acetate (72:28, v/v) in terpenoids; toluene: acetone: formic acid (22:22:6) in phenolics and Butanol: acetic acid: water (4:1:5) in glycosides. After establishment, the plate was densitometrically assessed over wavelengths spanning from 300 to 500 nm, with the highest findings obtained at 417 nm (Dhamal and Thorat, 2024).

M. esculenta leaf extracts' antioxidant potential in ethanol was evaluated by quantitative experiments that took into account their capacity to scavenge the stable DPPH free radical. A 1,1-diphenyl-2-picrylhydrazyl reactive species free radical scavenging assay was applied to gauge the antioxidant potential of *M. esculenta* leaf extract. The oxidisable groups of synthetic or natural antioxidants were titrated quickly and accurately with DPPH. DPPH was produced in an ethanol solution (Shekhar *et al.*, 2021). The raw extracts of *M. esculenta* were combined with ethanol to yield a stock solution that contained 10 mg/ml.

The sample solutions' concentration of 100 ìg/ml was used. The test samples, which had a concentration of 125, 250, 500, 800 and 1000 μg/ml, respectively, were generated by decreasing the stock solution using ethanol. In each of these test tubes, *M. esculenta* leaf extracts were added to a newly prepared DPPH solution. After 20 min, the absorbances were measured at 517 nm. Additionally, ascorbic acid was administered as a positive control. The DPPH solution devoid of the sample solution served as the control. 95% ethanol was used as a blank (Manjula *et al.*, 2019). To determine the following percentage of DPPH radical scavenging:

$$\text{Inhibition of DPPH free radical} = \{1-(\text{Abs}/\text{Abc})\} \times 100$$

Where, Abc (control's absorbance) and Abs (sample solutions).

Gallic acid served as the reference standard for the Folin-Ciocalteu reagent, which was a tool to figure out the phenolic concentration. After

having been diluted with ethanol, the crude extract was incorporated with the Folin-Ciocalteu reagent (Lawag *et al.*, 2022). Once Na, COƒ was added the resulting mixture was incubated, the percentage of the phenolic content as gallic acid equivalent (GAE) per gram of extract was calculated via measurement of the absorbance at 750 nm. In addition, using the colorimetric aluminium chloride method, flavonoids were identified. To assess the total flavonoid content, quercetin was utilized.

To assess *M. esculenta* leaf extracts' antibacterial efficacy against bacterial strains: MTCC and IMTECH, fresh cultures were generated using Muller-Hinton broth (MHB) and cultured at 37°C for bacteria (Kabra *et al.*, 2019). The reaction mixture included various strengths of ethanolic leaf extracts as well as 1% BSA (aqueous solution). 1 N HCl was employed to alter the pH of the resulting solution. After being heated for 15 min at 37°C and subsequently 15 min at 60°C, the samples were left to cool. The resulting turbidity was assessed at 660 nm. Puspal *et al.* (2017) used to calculate the percent inhibition of protein: Per cent inhibition of protein = 100 x (Absorbance of the ethanolic leaf extract – Control absorbance).

One ml of ethanolic leaf extract (at varying concentrations) and 1 ml of 10% red blood cell suspension (made from HRBCs that had been anticoagulant-treated and three times rinsed with normal saline) made up the reaction mixture (2 ml). Instead of using plant extract as the control, saline was added. The conventional medication used as a positive control was acetaminophen. After centrifuging the samples for 8 min at 3000 rpm and incubating them for 25 min at 56°C, the absorbance of the supernatant was measured at 560 nm (Puspal *et al.*, 2017).

In phenol red-free RPMI supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated foetal bovine serum, the J774 macrophage cell line was cultivated at 37°C with 5% CO, . After being cleaned with RPMI media, cells were separated using 0.25% trypsin-EDTA. After being seeded in a 96-well plate at a density of 10^5 cells/well, the cells were incubated for 24-48 h at 37°C with 5% CO_2. Following aspiration, new FBS-free RPMI media was incorporated into each and every well's media. A total of 100 µl was made in each well of a microtiter plate using varying amounts of *M. esculenta* leaves (125-1000 µg/ml) in RPMI containing FBS. For 24-48 h, the cells were co-incubated with 1 µg/ml of LPS (along with and without leaf extract) and then centrifuging to collect the supernatant for estimation of NO and estimation of TNF alpha cytokine by Elisa technique. After 48 h, the formazan crystals were produced using MTT dye (2.5 mg/ml; 10 µl) and dissolved in dimethyl sulfoxide, and the cytotoxicity was evaluated (Surin *et al.*, 2017). At 570 nm, the measurements were taken. For every sample, three wells were used.

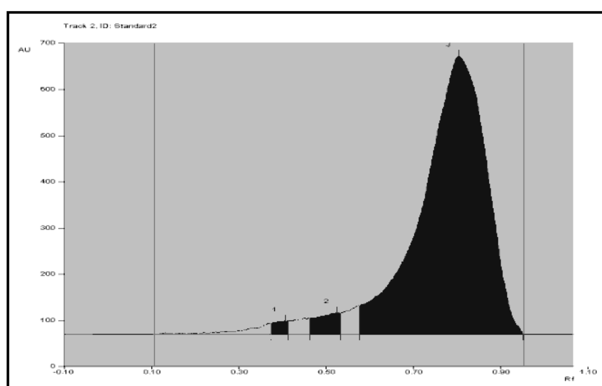
Nitrite, a stable oxidised product of NO, was identified in cell culture surroundings using Griess reagent. 50 µl of the test culture's supernatant and 50 µl of 1% (w/v) sulphuric acid in 5% (v/v) phosphoric acid were combined on a 96-well plate, and the mixture was then allowed to sit at room temperature for 10 min. 50 µl of 0.1% (w/v) N-1-naphthyl ethylenediamine HCl in distilled water was then added, and the mixture was allowed to sit at room temperature for 10 min. Using a microplate reader, the optical density at 540 nm was determined (Mfotie *et al.*, 2017). By comparing the NO concentration to a standard calibration of $NaNO_2$ (0-100 µM), it was determined.

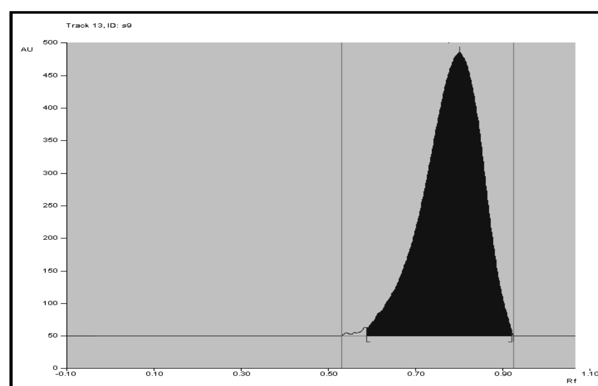
As per the directions provided by the manufacturer, a readily accessible ELISA kit was utilized for calculating how much of TNF alpha/IFN gamma was in the culture supernatant. Standards and samples (100 µl each) were loaded into wells that were coated, and the wells were then incubated for 2 h at 37°C. Following washing to eliminate any unbound materials, biotin-conjugated anti-TNF alpha/anti-IFN gamma antibody was added and incubated at 37°C for 1 h. After giving the wells another wash, the streptavidin-HRP conjugate was added, and they were left to incubate for half an hour at room temperature. TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was applied following the last wash, and it was left to develop colour for up to 15 min in dark conditions and then measured at 450 nm.

RESULTS AND DISCUSSION

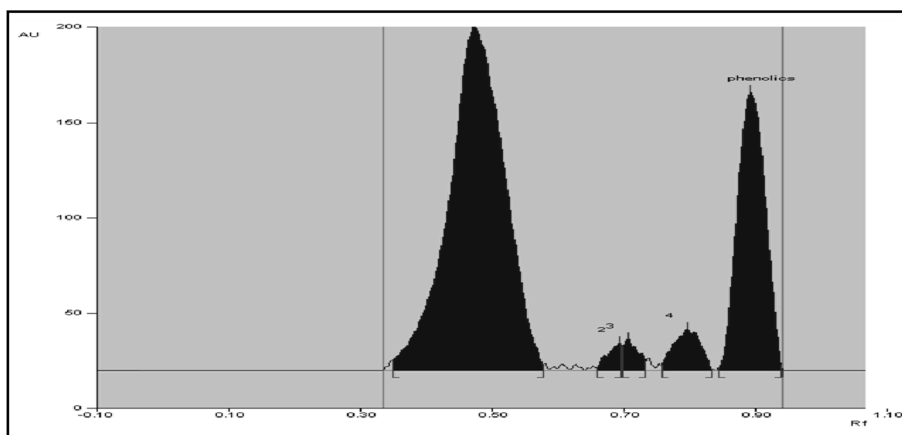
Antioxidants are essential substances that aid in the human system's defense against destructive free radicals, alleviating oxidative stress and averting cellular damage. An abundance of naturally occurring compounds, including phenolics, flavonoids, alkaloids,

tannins and vitamins, make medicinal plants one of the best suppliers of natural antioxidants. As per the results of this study, the ethanolic extract of *M. esculenta* leaves revealed the existence of terpenoids, phenolics and glycosides, as shown in Fig. 1a, b and c. By altering the body's enzymatic and non-enzymatic antioxidant systems, these naturally occurring chemicals not only scavenge free radicals but also strengthen defenses. Due to their effectiveness, low toxicity and accessibility in comparison to synthetic antioxidants, plants with medicinal properties are growing more popular as alternative treatments on a global scale. The function of medicinal plants in fostering health, slowing the course of disease and offering a sustainable method of treating problems linked to oxidative stress is further highlighted by the combination of contemporary pharmacological research with traditional knowledge. The study's results significantly enhanced the

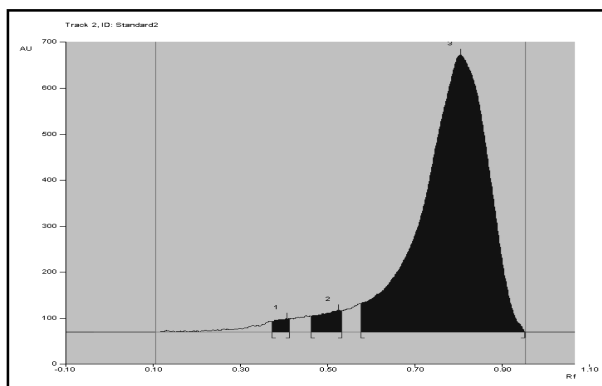


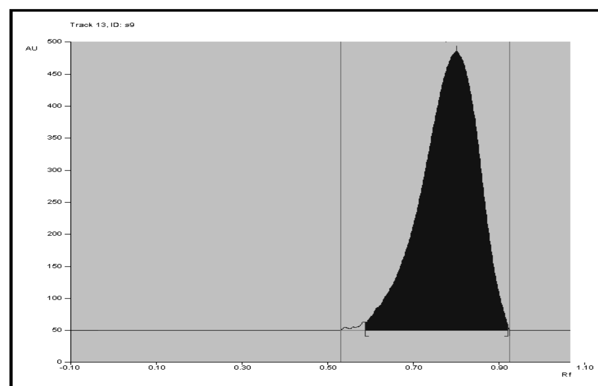


(a) Terpenoid peaks using solvent system [N hexane: ethyl acetate, (72:28)].



(b) Phenolics peaks using solvent system [toluene: acetone: formic acid [22:22:6]].





(c) Glycoside peaks using butanol: acetic acid: water (4:1:5)

Fig. 1. HPTLC analysis of secondary metabolites from *Myrica esculenta*.

antioxidant activity of the leaf extract, as demonstrated by the DPPH assay. This increase in free radical scavenging potential can be directly attributed to the elevated levels of phenolic and flavonoid compounds present in the extract. Phenolics and flavonoids are well-recognised natural antioxidants that act by donating hydrogen atoms or electrons to neutralise free radicals, thereby preventing oxidative damage. Their synergistic effect not only strengthens the radical scavenging capacity but also contributes to the overall improvement in antioxidant activity. The ethanolic extract of *M. esculenta* exhibited exceptional *in vitro* DPPH radical scavenging activity in a manner that was dose-dependent. L-ascorbic acid, the standard, had noticeably greater DPPH radical scavenging properties than did any of the ethanolic plant extracts under consideration (Table 1).

Table 1. *In vitro* DPPH scavenging activities of ethanolic extract of *Myrica esculenta*

Concentration (µg/ml)	DPPH scavenging activity (% inhibition)	
	L-ascorbic acid	*Myrica esculenta*
125	48.26±0.78	29.82±0.72
250	60.62±0.56*	44.84±1.02
500	71.42±0.94**	53.12±0.66
800	82.64±0.88**	62.84±0.98*
1000	92.18±1.22***	67.34±1.14**

The values are expressed as mean±SEM.

The extract displayed noticeably greater DPPH radical scavenging capabilities than the control group at all doses examined. This study also established the extract concentrations needed to scavenge 50% of the DPPH radicals (IC50). In contrast, extract of *M. esculenta* and the standard (ascorbic acid) had respective IC50 values of 0.23 and 0.068 mg/ml.

The amount of total phenols in the ethanolic extract of *M. esculenta* was assessed in the present investigation. The ethanolic extract had a substantially raised total phenolic content (48.54±1.34 mg of GAE/g), based on reported experimental data. However, an investigation of the total flavonoid amount in the ethanolic extract of the plant extract based on the study suggested that *M. esculenta* had a much more substantial total flavonoid content (41.38±0.86 mg of QE/g). Hence, the findings suggested that the enhanced DPPH activity was mainly due to the higher accumulation of phenolic and flavonoid content in the extract. In other words, the extract of leaves was found to significantly enhance the phenolic and flavonoid content, which were well-known bioactive compounds responsible for antioxidant activity. The elevated levels of these compounds correlated with a strong scavenging potential in the DPPH assay, indicating the ability of the extract to neutralise free radicals and reduce oxidative stress.

Another investigation determined that the ethanolic extract of leaves from *M. esculenta* was significantly more effective against *Staphylococcus aureus* (MTCC 737) and *Pseudomonas aeruginosa* (MTCC 2582), with a zone of inhibition of 9.2±0.12 and 10.4±0.18, respectively (Fig. 2 and Table 2). All bacterial species' observable growth was suppressed in the wells that contained extracts of *M. esculenta* at concentrations of 125-250 µg/ml. Following a 24-h incubation period at 37°C, the diameter of the zone of inhibition was measured in millimeters. The absence of inhibition in the solvent control (ethanol) demonstrated that the extract's bioactive components were the cause of the activity. At some concentrations, the efficacy was at par with that of conventional antibiotics, indicating that it was developed into a natural antibacterial agent.

To further explore the mechanism of the ethanolic leaf extract's anti-inflammatory capabilities, protein denaturation (Fig. 3) and membrane stabilization of hRBCs (Fig.4) were addressed. The current study's findings showed that ethanolic leaf extract, at higher concentrations, successfully prevented heat-induced haemolysis and stabilized red blood cell membranes. It also substantially reduced protein denaturation. Research is necessary to determine if the ethanolic leaf extracts prevent neutrophils' lysosomal material from being released at inflammatory locations (Puspal *et al.*, 2017). Neutrophils' lysosomal components contained bactericidal as well as protease enzymes, which, when released extracellularly, increased tissue inflammation and damage (Puspal *et al.*, 2017).

Interestingly, in cell culture supernatants, the extract was observed to decrease nitric oxide (NO) production as well as the pro-inflammatory cytokine tumour necrosis factor (TNF). The results of these studies revealed that leaf ethanolic extract inhibited the NO production when cell culture supernatant was collected

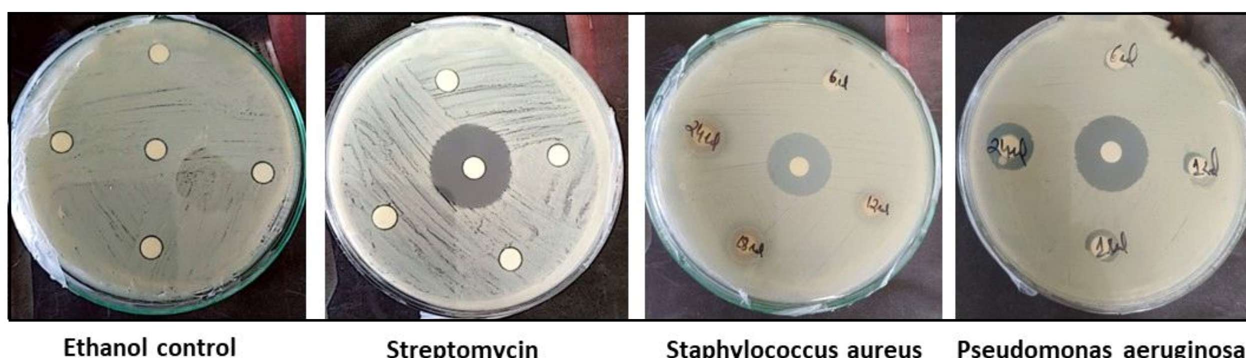


Fig. 2. Agar diffusion method using disk-diffusion method of *Myrica esculenta* leaf extract.

Table 2. Antimicrobial activity

Samples	Concentration of extract µg/ml (50 µl)	*Staphylococcus aureus* (MTCC 737)	*Pseudomonas aeruginosa* (MTCC 2582)
Leaves (*Myrica esculenta)*	31.25	3.5±0.1	4.5±0.1
	62.5	7.5±0.08	7.5±0.2
	125	8.6±0.18*	9.5±0.12*
	250	9.2±0.12**	10.4±0.18**
Streptomycin	50	11.4±0.22***	14.4±0.18***

*P<0.05, **P<0.01 and ***P<0.001 using one-way ANOVA.

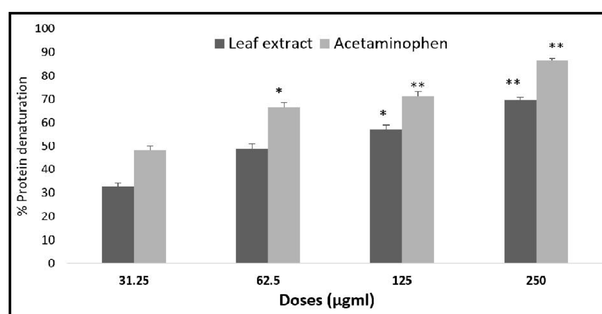


Fig. 3. Protein denaturation assay. Values presented as mean±S.E.
*P<0.05, **P<0.01 and ***P<0.001 using one-way ANOVA.

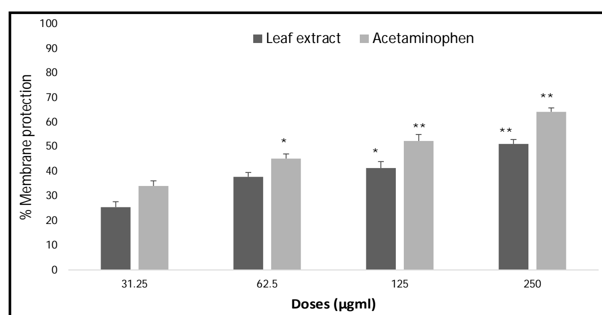


Fig. 4. Membrane stabilization assay. Values presented as mean±S. E.
*P<0.05 and **P<0.01 using one-way ANOVA.

after exposure (24 and 48 h, LPS stimulated) to different concentrations as compared to the control (Fig. 5). Acetaminophen was used as a standard for these studies and inhibited nitric oxide as compared to extract and control. Overall, leaves ethanolic extract showed anti-inflammatory activity. Similarly, cell culture supernatant was collected after centrifuging the samples (i.e. J774 cell lines exposed to different concentrations of ethanolic leaf extract) for the estimation of TNF alpha and IFN gamma (at different time intervals i.e. 24 and 48 h, LPS stimulated) by the ELISA method. The results of these studies showed that cell culture supernatant of higher concentration showed a decline in TNF alpha production as compared to the control (Fig. 6).

Since NO, TNF alpha and IFN gamma are key mediators in inflammatory pathways, their reduction suggests that the leaf extract possesses potent anti-inflammatory effects. Thus, the dual action (NO and inflammatory cytokines) of enhancing antioxidant defenses while simultaneously suppressing inflammatory mediators demonstrate that the leaf extract exerts both antioxidant and anti-inflammatory activities, making it a promising candidate for therapeutic or nutraceutical applications. Since the J774 macrophage cell line might generate NO whenever provoked by triggers of inflammation like lipopolysaccharides (LPS) or cytokines, it is frequently applied for evaluating NO generation. The extract of leaves was given to J774 macrophage cells during this research in order to figure out its influence on NO production. Contrary to the outcomes, the leaf extract substantially diminished NO production,

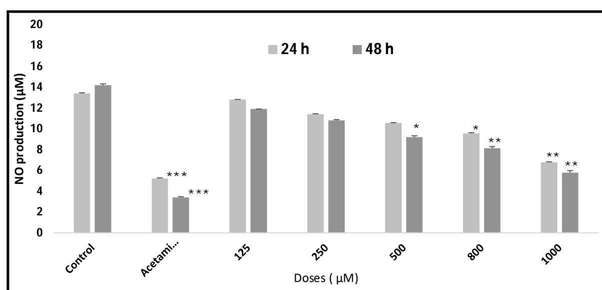
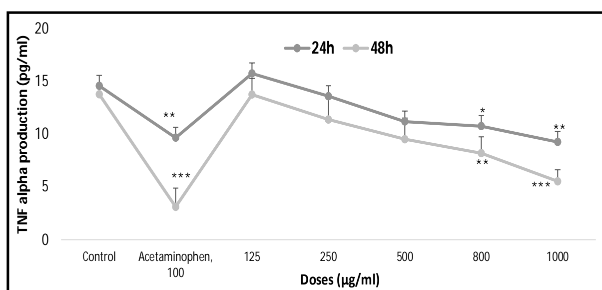
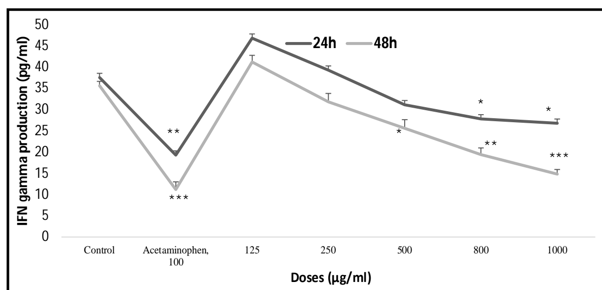


Fig. 5. NO production from J774 macrophage cell culture supernatant at different time intervals i.e. 24 and 48 h which was determined through Griess reagent. Values were expressed in the form of Mean±S.E.



(a) TNF alpha estimation



(b) IFN gamma production

Fig. 6. TNF alpha and IFN gamma production from J774 macrophage cell culture supernatant at different time intervals i.e. 24 and 48 h which was determined through ELISA. Values were expressed in the form of Mean±S.E.

implying the ingredient may have anti-inflammatory attributes. As NO created from macrophages serves as crucial for inflammatory processes to occur, its suppression indicated the extract influenced macrophage activation and their expulsion of inflammatory mediators. In simple terms, the formation of nitric oxide was significantly reduced by leaf extract, and this change was countered by a fall in TNF-á levels. This raised the possibility of a relationship between TNF-á and nitric oxide, two substantial pro-inflammatory mediators generated by activated macrophages. A reduction in TNF-á may inhibit the induction of iNOS, which would reduce the generation of NO. On the other hand, lower NO levels may suppress inflammatory signalling, which would lower the production of TNF-á. Based on these results, the leaf extract had anti-inflammatory properties, either via modifying the NO and TNF alpha pathways in macrophages. Similarly, the results revealed a decrease in NO generation, suggesting a potential impairment in interferon-gamma activity. IFN-gamma is a vital cytokine that encourages macrophages and prompts the emergence of inducible nitric oxide synthase, and this resulted in an output of NO (Bouyahya *et al.,* 2018). Thus, a decrease in NO levels frequently indicated that IFN-gamma-mediated macrophage activation had been restrained. In the current investigation, ELISA analysis verified that the treated samples' IFN-gamma concentration was significantly lower than that of the control group. This decrease was correlated with lower NO levels, indicating that the leaf ethanolic extract limited the inflammatory response by blocking the generation of IFN-gamma.

At higher doses of ethanolic leaf extract, there was continuously declining (dose-dependent) in the concentration of J774 macrophage cell lines as compared to control (Fig. 7). The cytotoxic impact of the extract on macrophage cell line had been shown by the dose-dependent decrease in cell viability that followed treatment with different doses of the extract. The reduction in cell viability implied that the extract's bioactive ingredients prevented the growth of cells or triggered their death at higher concentration. These results indicate the leaf extract's potential usefulness as an herbal source of therapeutic agents by underlining its potential anti-inflammatory characteristics.

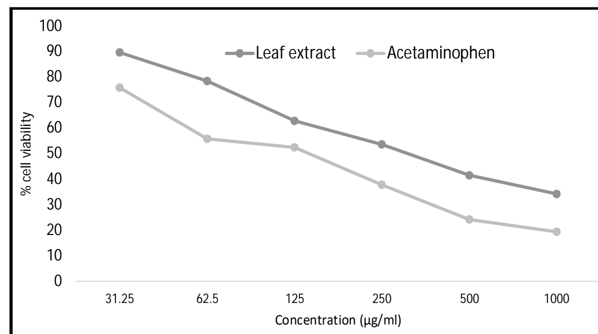


Fig. 7. Cytotoxicity assay using J774 macrophage cell lines and results were expressed in terms of cell viability percentage.

CONCLUSION

HPTLC was implemented for identifying the plant constituents and demonstrated relatively significant phenolic and flavonoid content. The extracts employed in this investigation have anti-inflammatory and antioxidant properties. This study found that *Myrica esculenta* leaf extract in ethanol had an elevated amount of phytoconstituents and strong anti-inflammatory and antioxidant properties (inhibition of NO and TNF alpha generation). To work on various solvent systems and ascertain their immunopharmacological effects, more research is needed.

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