



Comparative phytochemical and antimicrobial study of *Moringa oleifera* lam. in two different seasons

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Abstract

This study evaluated variations in phytochemical composition and antimicrobial activity of *Moringa oleifera* leaves collected in two different seasons, Karkidakam and non-Karkidakam from Kerala, India. Qualitative and quantitative phytochemical analyses were done using methanolic extracts, while n-hexane extracts were evaluated using GC-MS. Antimicrobial activity was assessed against selected bacterial and fungal pathogens using agar well diffusion and radial growth inhibition assays. Results showed higher levels of alkaloids, tannins and phenolic compounds in non-Karkidakam samples, whereas flavonoids are found to be comparatively higher during the Karkidakam season. GC-MS analysis revealed seasonal differences in fatty acids and their derivatives, with hexadecanoic acid and octadecatrienoic acid as major constituents. Antibacterial activity was observed mainly in Non-Karkidakam extracts, while Karkidakam extracts showed stronger antifungal activity. The study demonstrates that seasonal variation significantly influences phytochemical composition and antimicrobial properties of *Moringa oleifera* leaves.

Keywords: *Moringa oleifera*, seasonal variation, phytochemical screening, GC-MS analysis, antimicrobial activity, karkidakam

Introduction

Plants have always been vital for mankind, irrespective of the era and area all over the globe, since the beginning of life (Mahmood *et al.*, 2010) [11]. Various types of plants have been used for several centuries worldwide, not only as dietary supplements but also as traditional treatments for many diseases (Jung, 2014) [8]. WHO has stated that herbal or medicinal plants are the best source to obtain a variety of drugs (Olayemi *et al.*, 2016) [12].

Phytochemicals have an important role in curing diseases and are responsible for providing colour, flavour, and aroma to fruits and vegetables. Phytochemical analysis is a technique developed for the analysis of various constituents present in plants. Antimicrobial assays are an important tool to test and screen the inhibitory effects of myriad compounds against microorganisms before establishing their inhibitory spectra (Bailey, 2013) [3].

Moringa oleifera Lam. is a small deciduous tree belonging to the family Moringaceae. It is also called drumstick tree, horseradish tree, ben oil tree and miracle tree. It is a small, graceful, deciduous plant with thin foliage and can grow up to 8m in height (Keay, 1989) [9]. It is grown worldwide in the tropics and sub-tropics of Asia and Africa and comprises 13 species. The most cultivated species is *Moringa oleifera* (Rockwood *et al.*, 2013) [13].

Moringa oleifera is a plant that has been used for centuries in traditional medicine due to its numerous health benefits. It has tremendous nutritional and medicinal properties. However Many suggest not consuming *Moringa* leaves during Karkidaka masam, which is the month where there is heavy rain and dark sky throughout. Chances are that during Karkidaka Masam, toxins would be released into its leaves.

Phytochemical composition in plants is not constant and can vary significantly depending on environmental conditions such as temperature, rainfall, light intensity, soil nutrients, and seasonal changes. Seasonal variation plays an important role in regulating the synthesis and accumulation of

secondary metabolites, which in turn may influence the biological efficacy of plant extracts. Several studies have demonstrated that seasonal differences can affect both the qualitative and quantitative phytochemical profiles of medicinal plants, thereby altering their antimicrobial potential (Yang *et al.*, 2018; Verma & Shukla, 2015) [21, 23]. Although numerous studies have reported the phytochemical constituents and antimicrobial activity of *Moringa oleifera*, comparative studies focusing on seasonal variation in phytochemical composition and corresponding antimicrobial efficacy remain limited. Understanding how seasonal factors influence the bioactive profile of *Moringa oleifera* is essential for optimising its medicinal use and ensuring consistency in therapeutic applications. Therefore, the present study aims to conduct a comparative phytochemical and antimicrobial analysis of *Moringa oleifera* collected during two different seasons, with the objective of evaluating seasonal variations in phytochemical constituents and assessing their influence on antimicrobial activity against selected microbial pathogens.

Materials and Methods

1. Plant Material Collection and Preparation

Fresh leaves of *Moringa oleifera* Lam. were collected during two different seasons (July and January) from Kadannappalli, Kerala. The collected samples were washed thoroughly with running tap water, followed by distilled water to remove debris and dust. The leaves were shade-dried at room temperature (25–28 °C) for 4–5 weeks until constant weight was obtained. The dried material was powdered using a mechanical grinder and stored in airtight containers until further analysis.

2. Preparation of Plant Extracts

Methanolic Extract

Dried leaf powder (4 g) from each seasonal sample was extracted with 40 mL of methanol using a rotary shaker. The

mixture was filtered and centrifuged, and the supernatant was collected. The crude extract was stored in sterile glass bottles under refrigerated conditions until further use.

3. Aqueous Extracts (for Antimicrobial Assay)

Two aqueous extracts were prepared:

1. **Cold water extraction:** One gram of powdered sample was suspended in 10 mL distilled water and shaken at 27 °C for 15 min. The mixture was centrifuged at 5000 rpm for 10 min at 10 °C, and the supernatant was collected.
2. **Hot water extraction:** One gram of powdered sample was boiled in 10 mL distilled water for 5 min, centrifuged as above, and the supernatant was collected.

3. Qualitative Phytochemical Screening

Preliminary phytochemical screening of methanolic extracts was carried out to detect the presence of alkaloids, tannins, flavonoids, phenols, steroids, saponins, terpenoids, phlobatannins, coumarins, cycloglycosides, and quinones using standard procedures described by Harborne (1998) [7]. Colour changes or precipitate formation were recorded as indicative of the presence of respective phytoconstituents.

4. Quantitative Phytochemical Analysis

Total Alkaloid Content

To 1 ml of test extract, 5 ml, pH 4.7 phosphate buffer was added and 5 ml of BCG (Bromocresol Green) solution and shake a mixture with 4 ml of chloroform. The extract was collected in a 10 ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex was measured at 470nm against blank prepared as above, but without the extract. Atropine is used as standard to compare the assay with Atropine (Shamsa *et al.*, 2008) [16].

Total Tannin Content

From 1 ml of the sample extract concentration, 1 mg/ml was taken in a test tube. The volume was made up to 1 ml with distilled water and add 1 ml of water serves as the blank. To this 0.5ml of Folin phenol reagent followed by 5ml of 35% sodium carbonate was added and kept at room temperature for 5 min. Blue colour was formed and the colour intensity was read at 640nm. A standard graph Gallic acid was plotted from which the tannin content of the extract was determined (Singleton & Rossi, 1965) [18].

Total Flavonoid Content

A volume of 0.25 ml of the sample was diluted to 1.25 ml with distilled water. 75ul of 5% sodium nitrate was added and after 6 min., 10% Aluminium chloride solution was added. After 6 min. Incubation at room temperature 2 ml of 1M sodium hydroxide was added to the reaction mixture. Immediately, the final volume was made up to 10 ml with distilled water. The absorbance of the mixture was measured at 510 nm against a blank spectrophotometrically (Chang *et al.*, 2002) [5].

Total Phenolic Content

0.5ml of each sample were introduced into test and mixed with 2 ml of 10 fold dilute folin ciocalteu reagent and 4ml

of 7.5% sodium carbonate. The tubes were covered with parafilm and it was incubated at room temperature for 30 min. with intermittent shaking, and the absorbance was taken at 765nm against using methanol as blank. Total phenolic content was calculated by the standard regressive curve of gallic acid, and the results were expressed as gallic acid equivalents (Singleton *et al.*, 1999) [19].

All spectrophotometric measurements were performed using a UV–Visible spectrophotometer.

GC–MS Analysis

The bioactive compounds present in the plant extract were identified using Gas Chromatography–Mass Spectrometry (GC–MS) analysis (Shimadzu GCMS QP2020 NX) equipped with an Rtx-5Sil MS column (30 m × 0.25 mm × 0.25 µm). Helium was used as the carrier gas. The injector temperature was maintained at 280 °C, and mass spectra were recorded under electron impact (EI) mode over a scan range of m/z 40–550. Identification of compounds was performed by comparing mass spectra with those available in the NIST (2020) library.

For GC–MS analysis, 1 g of sample was extracted with 50 mL n-hexane using Soxhlet extraction for 2 h. The extract was evaporated under nitrogen and reconstituted in 1mL of hexane prior to injection.

Antimicrobial Activity

Antibacterial activity was evaluated against *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Proteus mirabilis* using the agar well diffusion method (Valgas *et al.*, 2007) [22]. Antifungal activity was tested against *Aspergillus fumigatus* and *Penicillium* sp. using a radial growth inhibition assay (Balouiri *et al.*, 2016) [4].

Antibacterial Assay

Sterile nutrient agar medium seeded with test bacteria such as *Enterococcus faecalis*, *E.coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Proteus mirabilis* was poured on the surface of sterile petri dishes and allowed to set. Wells of 6mm diameter were punched with the help of a cup borer, and the extract was loaded into the wells. The bacterial plates were then incubated at 37°C in an incubator. The zones of microbial growth inhibition were measured and recorded at the end of the incubation period.

Antifungal Assay

To determine the susceptibility of moulds to the plant extract, mould strains such as *Aspergillus fumigatus* and *Penicillium* spp. were chosen for study. The strains were point-inoculated at the centre of the PDA plate. After obtaining a growth of 10-15mm diameter, a sterile filter paper disk of 6mm diameter impregnated with the plant extract was carefully placed at a distance of 5mm from the growth. After overnight incubation at room temperature, the plates were observed for the presence of growth inhibition areas near the disk.

Result and Discussion

The following are the observations of preliminary phytochemical screening of leaf extract of *Moringa oleifera*.

1. Qualitative Phytochemical Analysis

Table 1: Qualitative analysis of major phytochemical constituents in *Moringa oleifera* leaf samples

Phytochemicals	Name of Reagent	Observation	Sample1(Non-karkidakam)	Sample2 (Karkidakam)
Alkaloids	Mayer's reagent	Cream coloured precipitate	+++	+
Tannins	1% lead acetate	Yellowish precipitate	+++	++
Steroids	1ml acetic acid, 1ml chloroform, 0.5ml sulphuric acid	Violet to blue-green colour	-	-
Flavonoids	1ml sulphuric acid	Orange colour	+++	++
Phenols	3% ferric chloride	Deep blue colour	+++	++
Saponins	4ml distilled water	Appearance of foam	+++	+
Terpenoids	2ml acetic acid, 1ml sulphuric acid	Blue-green ring	+	-
Phlobotannins	1ml of 1%HCL	Red precipitate	-	-
Coumarins	3ml of 10% NaOH	Yellow colour	++	++
Cycloglycosides	1ml acetic acid, 1%ferric chloride	Greenish ring	++	++
Quinones	4ml of HCL	Yellow coloured precipitate	++	++

(+++) High presence of phytochemicals, (++) Moderate presence of phytochemicals, (+) Less presence of phytochemicals, (-) Absence of phytochemicals

2. Quantitative Phytochemical Analysis

Table 2: Quantitative analysis of major phytochemical constituents in *Moringa oleifera* leaf samples

Sample	Alkaloid (mg AE/g extract)	Tannin (GAE/g extract)	Flavonoids (mg QE/g extract)	Phenol (mg GAE/g extract)
Sample 1 (Non-Karkidakam)	4.07	0.023	3.66	4.07
Sample 2 (Karkidakam)	1.94	0.020	4.20	3.18

3. GC MS Analysis

GC-MS analysis revealed distinct seasonal variations in the chemical composition of *Moringa oleifera* leaf extracts collected during Non-Karkidakam and Karkidakam seasons.

A total of 20 compounds were identified in the Non-Karkidakam sample, whereas 25 compounds were detected in the Karkidakam sample.

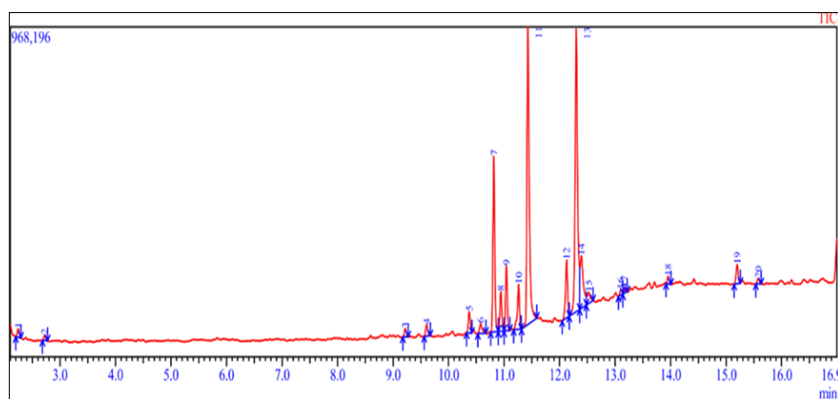


Fig 1: GC-MS Chromatogram profile of *Moringa* leaf Sample1 (Non-Karkidakam)

Table 3: Major chemical components in the *Moringa* leaf of Sample1 (Non-Karkidakam)

Retention time (min)	Compound identified	Area (%)	Chemical class
12.302	9,12,15-Octadecatrienoic acid (Z,Z,Z)	27.67	Fatty acid
11.430	n-Hexadecanoic acid	26.92	Fatty acid
10.816	Neophytadiene	12.70	Alkene
12.394	Octadecanoic acid	6.42	Fatty acid
12.129	11,14,17-Eicosatrienoic acid, methyl ester	4.73	Fatty acid ester
11.043	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	4.52	Alcohol
11.263	Hexadecanoic acid, methyl ester	3.76	Fatty acid ester

In the Non-Karkidakam sample, fatty acids constituted the dominant class of compounds. The major constituents were 9,12,15-Octadecatrienoic acid (27.67%), n-Hexadecanoic acid (26.92%), Neophytadiene (12.70%), and Octadecanoic acid (6.42%). Fatty acid esters such as 11,14,17-Eicosatrienoic acid, methyl ester and Hexadecanoic acid, methyl ester were also detected in moderate proportions.

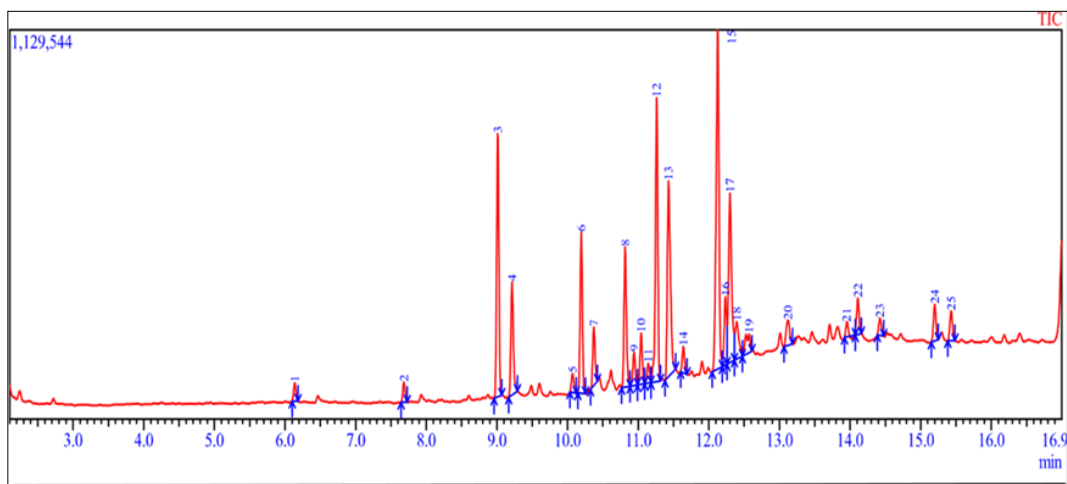


Fig 2: GC-MS Chromatogram profile of *Moringa* leaf Sample2 (Karkidakam)

Table 4: Major chemical components in the *Moringa* leaf of Sample2 (Karkidakam)

Retention time (min)	Compound identified	Area (%)	Chemical class
12.127	11,14,17-Eicosatrienoic acid, methyl ester	16.73	Fatty acid ester
11.262	Hexadecanoic acid, methyl ester	11.89	Fatty acid ester
11.430	<i>n</i> -Hexadecanoic acid	10.43	Fatty acid
9.012	Dodecanoic acid, methyl ester	10.18	Fatty acid ester
12.300	9,12,15-Octadecatrienoic acid (Z,Z,Z)	9.35	Fatty acid
10.195	Methyl tetradecanoate	6.26	Fatty acid ester
10.816	Neophytadiene	5.88	Alkene
9.214	Dodecanoic acid	4.90	Fatty acid

The Karkidakam sample showed a comparatively higher abundance of fatty acid esters, with 11,14,17-Eicosatrienoic acid, methyl ester (16.73%), Hexadecanoic acid, methyl ester (11.89%), and Dodecanoic acid, methyl ester (10.18%) as major components. Fatty acids including *n*-Hexadecanoic acid (10.43%) and 9,12,15-Octadecatrienoic acid (9.35%)

were also prominent. The alkene Neophytadiene was detected in both seasonal samples, though with slightly lower abundance during Karkidakam.

4. Antimicrobial analysis

Table 5: Antibacterial activity of two samples of *Moringa oleifera* (Non-karkidakam and Karkidakam)

Sl.No.	Bacteria used	Non-Karkidakam <i>Moringa</i> leaves extract	Karkidakam <i>Moringa</i> leaves extract	Diameter of Inhibition Zone
1	<i>Enterococcus faecalis</i>	Shows activity	No action	14mm
2	<i>Staphylococcus aureus</i>	Shows activity	No action	14mm
3	<i>Klebsiella pneumoniae</i>	Shows activity	No action	15mm
4	<i>Escherichia coli</i>	No action	No action	-
5	<i>Proteus mirabilis</i>	No action	No action	-

The Karkidakam and Non-Karkidakam *Moringa* leaf extracts were prepared using cold and boiled extracts. So there are four extract such as Karkidaka (H), Karkidaka(C), Non-Karkidakam(H) and Non-Karkidakam (C). Five bacteria, such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus mirabilis* were used. Only Non-Karkidakam Cold extract showed

antibacterial activity against three bacteria, such as *Enterococcus faecalis*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. No activity was shown against *E.coli* and *Proteus mirabilis*. Karkidakam (H), Karkidakam(C), and Non-Karkidakam (H) extracts showed no activity. So, it can be concluded that Non-Karkidakam *Moringa* leaves have antibacterial activity.

Table 6: Antifungal activity of two samples of *Moringa oleifera* (Non-karkidakam)

Sl.No.	Moulds used	Non-Karkidakam <i>Moringa</i> leaves extract	Karkidakam <i>Moringa</i> leaves extract
1	<i>Aspergillus fumigatus</i>	No action	Shows activity
2	<i>Penicillium</i> sp.	No action	Shows activity

The Mold strains such as *Aspergillus fumigatus* and *Penicillium* sps. were taken. Results showed that Inhibition of growth near Karkidakam hot extract were showed by *Penicillium* sps. and Inhibition of growth near both

Karkidakam hot and cold extracts were showed by *Aspergillus fumigatus*. No activity were shown by Non-karkidakam leaf extracts. So, it is concluded that Karkidakam *Moringa* leaf had Antifungal activity.

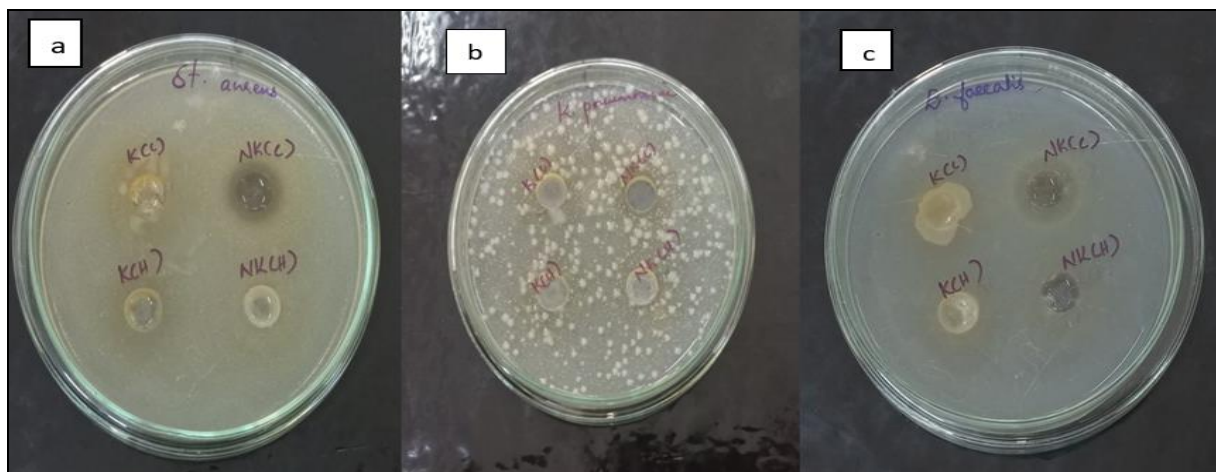


Fig 3: Antibacterial activity shown against (a) *Staphylococcus aureus*, (b) *Staphylococcus aureus*, (c) *Enterococcus faecalis*

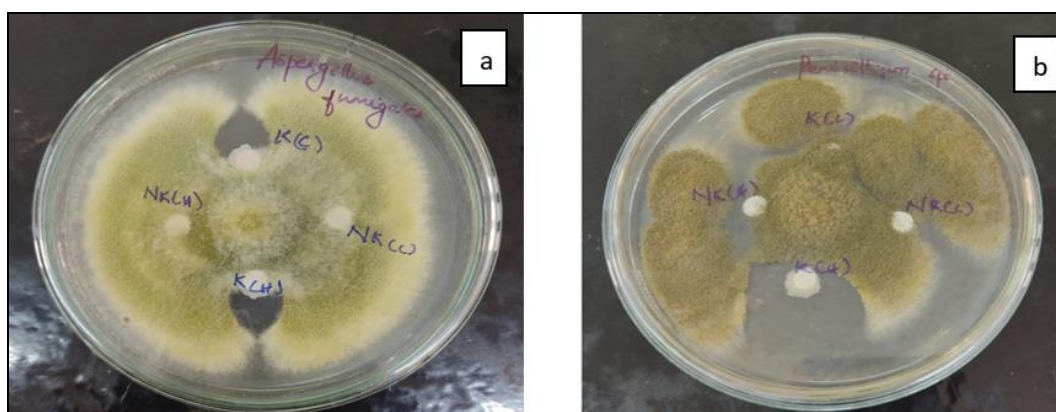


Fig 4: Antibacterial activity shown against (a) *Staphylococcus aureus*, (b) *Staphylococcus aureus*, (c) *Enterococcus faecalis*

Discussion

Moringa oleifera Lam. is a multipurpose tropical tree. It has been used as a system of Ayurvedic and Unani medicine for centuries and has a wide range of nutritional and bioactive compounds (Keay, 1989)^[9]

Evaluation of *Moringa oleifera* leaves from two different seasons (Karkidakam and non-Karkidam) for their phytochemical composition and antimicrobial activity revealed noticeable differences in their respective profiles. This indicates that environmental factors may influence the synthesis and accumulation of secondary metabolites in the plant.

Qualitative phytochemical analysis indicates that several phytochemicals are more abundant in Non-Karkidakam leaf extracts than in Karkidakam extracts. Phytochemicals such as alkaloids, Tannins, Flavonoids, Phenols, and saponins are abundant in Non-karkidakam samples, while Steroids and phlobotannins are absent in both samples. Coumarins, Cycloglycosides and Quinones are present at a moderate level in both samples. Terpenoids are present in the least in Non-karkidakam, while not present in the Karkidakam leaf extract. These variations could indicate the influence of seasonal changes in the production of phytochemical constituents in *Moringa* leaves.

Quantitative phytochemical analysis gives the amount of phytochemicals present, and this further confirms these differences. Phenols are present more in Non-Karkidakam leaf extract (4.07) than in Karkidakam leaf extract (3.18). Likewise, Tannin (0.023) and Alkaloids (4.07) are present more in Non-Karkidakam leaf extracts. But, Flavonoids are present more in the Karkidakam sample (4.20) than in the

Non-Karkidakam (3.66). Variations in phytochemical constitution in plants across seasons have been reported in several medicinal plants and may be associated with variation in environmental conditions such as rainfall, temperature, light intensity, and plant physiological status (Soni *et al.*, 2015)^[20]

Phytochemicals such as flavonoids, alkaloids, tannins, and saponins play important roles in plant defence and have been associated with several pharmacological activities (Koche *et al.*, 2016)^[10]. Phenolic compounds and flavonoids are known for their antioxidant properties and their ability to protect plants against environmental stress (Ahlawat *et al.*, 2024; Shomali *et al.*, 2022)^[2, 17]. Alkaloids and tannins are also known to possess antimicrobial and defensive functions in plants (Tiku, 2018)^[21]. The presence of these compounds in *Moringa* leaves may therefore contribute to their medicinal value and biological activity.

The Foam test confirmed the presence of saponins in the Non -Karkidakam leaf extract. In plants, saponins serve as anti-feedants and to protect plants against microbes and fungi (Saha *et al.*, 2017)^[14]. Similarly, tannins are recognised for their astringent properties and have been traditionally used in treatments of wounds, ulcers, and inflammatory conditions (Fraga-Corral *et al.*, 2021)^[6]

The major compounds, their retention time and peak area percentage of Non-karkidakam and Karkidakam leaf extracts obtained from the GC-MS profile of methanolic extract of *M.oleifera* leaves are shown in Table 3 and Table 4, respectively. The major compounds found are mostly fatty acids, alkenes and fatty acid esters. Such compounds have been previously reported in *Moringa oleifera* leaves

and are known to contribute to various biological activities, including antimicrobial and antioxidant effects (Saini *et al.*, 2016) [15].

The present study showed antibacterial and antifungal activity of the leaf extract of *Moringa oleifera*. Five bacteria, like *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *E. coli*, and *Proteus mirabilis*, were taken, and results showed that Non-Karkidakam Cold extract showed antibacterial activity against three bacteria while no action was determined against *E.coli* and *Proteus mirabilis* (Table 5). Antifungal activity was shown by Karkidakam cold and hot extract against moulds like *Aspergillus fumigatus* and *Aspergillus* spp (Table 6). These results can indicate that seasonal variation may have an influence on the antimicrobial properties in addition to phytochemical composition.

According to some traditional beliefs in Kerala, India, leaves of *Moringa oleifera* should not be consumed during the Malayalam month of Karkidakam (mid-July to mid-August), which falls during the peak Monsoon season. The present study intended to examine whether seasonal changes have any effect on the phytochemical composition and antimicrobial activity of *Moringa* leaves. The results showed considerable differences between leaves collected during Karkidakam and non-Karkidakam. Non-Karkidakam leaves showed a comparatively higher amount of phytochemicals and showed greater antibacterial activity, while Karkidakam extracts showed higher antifungal activity. These differences can be attributed to environmental factors such as heavy rainfall, high humidity, and reduced sunlight during the season. However, further studies are required to understand whether these seasonal changes have any significant effect on the safety or nutritional benefits of consuming the leaves.

Conclusion

The present study intended to explore the phytochemicals present in the plant, its biochemical compounds and antimicrobial activity.

Moringa oleifera is a multipurpose plant with many uses. It has been used not only for food but also has many medicinal properties. The present investigation revealed the presence of phytochemicals such as Alkaloids, Flavonoids, Tannins, Phenols, etc., their amounts, biochemical compounds and antimicrobial activity in *Moringa* leaves present in two different seasons. Qualitative and Quantitative phytochemical analysis were performed to find the phytochemicals and their amounts.

Non-Karkidakam leaf extracts contain more phytochemicals and antimicrobial activity than Karkidakam leaf extract. Antifungal activity is more on Karkidakam leaf extract.

Several aspects related to this topic can be taken as research topics in future to know more about it, like the season of Karkidakam, how it affects *Moringa* plants and the scientific facts behind toxicity in *Moringa* leaves during this period.

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Conflict of interest

The authors declare no conflict of interest.

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