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COMPREHENSIVE PHYTOCHEMICAL PROFILING AND BIOLOGICAL ACTIVITY ASSESSMENT OF CITRUS MAXIMA LEAF EXTRACTS: INSIGHTS FROM GC-MS ANALYSIS

Priya K[1], Parimala Gnana Soundari Arockiam Jeyasundar[2*], Minisha Udayakumar[3], M.P. Ayyappa Das[4*]

[1,2*,3,4*,]Department of Biotechnology, RVS College of Arts and Science, Sulur, Coimbatore 641 402, Tamil Nadu, India.

[*]Corresponding Author:
Department of Biotechnology, Rathnavel Subramaniam College of Arts and Science (Autonomous), Coimbatore, Tamilnadu, India – 641402;

Email: ayyappadasrvs@gmail.com, ayyappadas@rvsgroup.com, apgs_1987@yahoo.co.in

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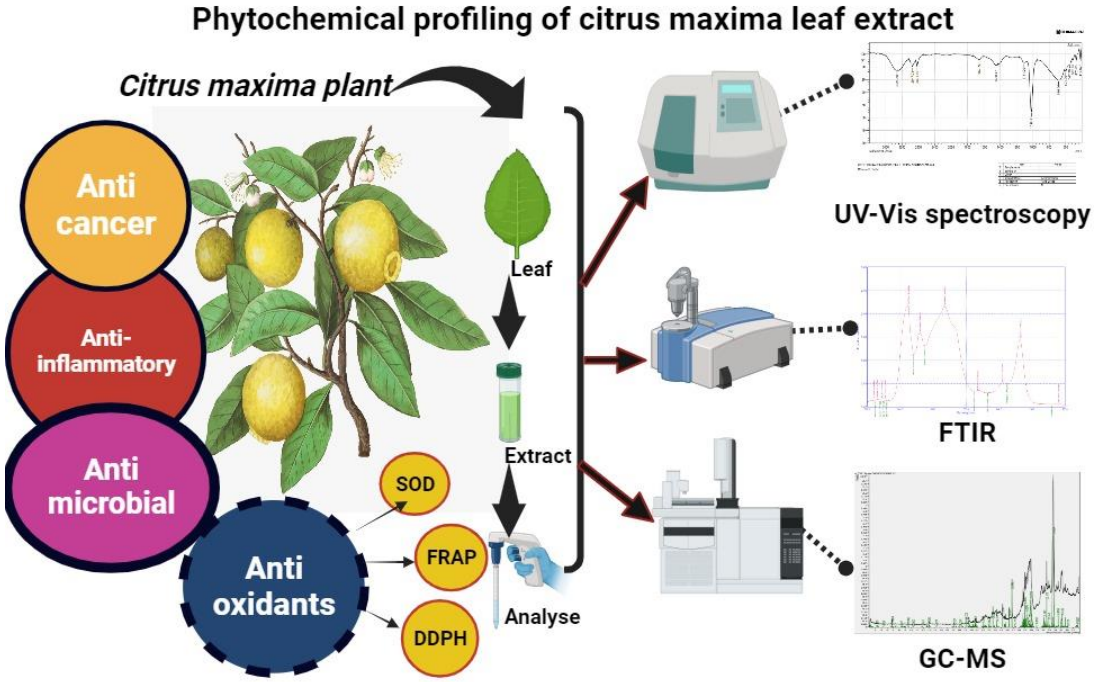
ABSTRACT:

Citrus is the most important genera of the Citrus family, having been cultivated round the globe and familiar for its nutritional, dietary, and therapeutic value for human health. This plant has enormous amounts of phytochemicals in all the parts including roots, shoots, leaves, fruits, and fruit peels. *Citrus maxima* are the largest fruit variety of citrus family and well known for its various ethnomedicinal significance and long been employed to treat various illnesses. It gained popularity due to its rich phytochemicals with medicinal significances, however only a few studies have been conducted in exploring the phytochemicals of their leaves. Hence, we hypothesis that the leaves of the *Citrus maxima* may be equally possessing a lot of phytochemicals which of clinical significance as like the earlier reported spectrum in its fruits and fruit peels. The main objective of our study focuses on analyzing the chemical compounds present in the leaves of the *Citrus maxima*, identifying the secondary metabolites and their biological activity. The leaf extract was thus obtained using methanol and water as solvents. The phytochemical compounds like phenols, saponins, terpenoids, flavonoids, steroids, quinines, steroids and sugar were present in the extracts. Total phenol, DPPH, FRAP, SOD and total antioxidant activity were found to be very significant. Further, the presence of various compounds in the leaf extracts were identified using UV Visible, FTIR, TLC and GC-MS. The plant extracts possessed antimicrobial and antifungal properties against common human pathogen. This study proves that the leaf extract holds potential antioxidant, biological and pharmacological significances.

Keywords: Citrus, leaf extract, phytochemicals, GC-MS, secondary metabolites, antioxidants

Graphical abstract



Highlights:

- Leaf extracts possessed antimicrobial and antioxidant activities.
- The phytochemical constituents of *Citrus maxima* leaf extracts were identified using GC-MS.
- The identified phytochemicals were found to be bioactive.
- The leaf extracts possessed anticancer agents suggesting their pharmaceutical properties

1. INTRODUCTION

Rutaceae is a group of flowering plants with approximately 150 genera and 1500 species. This family is known as the citrus family with the economically most important genera Citrus. It represents the major food crops that are produced in warm temperate and tropical climates around the world (Malacrida et al., 2012). The origin of Rutaceae and its subfamily Spathelioideae in the Late Cretaceous was likely to be from Africa and later diversified to other regions of the world (Appelhans et al., 2012). The Rutaceae members encompass mostly shrubs, trees and rare herbs, with some of being with thorns on the stem. The leaves of this family show simple, pinnate, trifoliate and pinnafied bearing pellucid or punctate glands. The flowers are found to be bisexual and actinomorphic, hypogynous, and rarely epigynous. The fruits are mainly schizocarp, berry, drupe, or hesperidium. Secretory cavities containing ethereal oils are present in many tissues, including the leaves and pericarp (Simpson, 2010). Citrus includes seventeen species; *C. lemon* (lemon), *C. aurantium* (bitter orange), *Citrus sinensis* (sweet orange), *C. reticulata* (mandarin orange, tangerine), and *C. paradise* (grapefruit) are few among them. The main by-products of citrus are pulp, peel and seeds (Lukitaningsih and Rumiyati, 2021). *Citrus maxima are* one of the largest fruit bearing citrus species, grown widely in temperate and tropical regions of Southeast Asia, China, Phillipines and Taiwan (Khan et al., 2018). This fruit is sour/sweet in taste and commonly called grapefruit, shaddock, pumelo, Chakotara etc. The leaves of this plant are primarily employed in treating seizures, ulcers, hemorrhages and especially skin disorders (Singh and

Singh, 2017). Earlier researches in this plant have documented the presence of various alkaloids, saponins and carbohydrates in the extracts of various plant parts (Sapkota et al.). These were found to contribute the potential activities including hypoglycemic, antitumor, analgesic, anti-inflammatory, antibacterial, antidepressive, anticonvulsant, muscle relaxant, hepatoprotective and antioxidants properties.

Besides all others, the current era majorly focuses on the antioxidant properties which are primarily needed for the other potent activities including fighting against cancer cells, a major threat these days (MU et al., 2019). Natural antioxidants which are of biological sources are highly preferential for human health as they could be able to reduce or prevent the impairment caused by the accumulation of ROS (reactive oxygen species) (Pisoschi et al., 2021). Rather on the scale of cost-efficiency, many of the synthetic alternatives are flooded into the market with better stability and activity (Augustyniak et al., 2010). However, their long-term consumption tags their side effects including allergic reactions, immunological issues, gastrointestinal problems, and difficulties to remove them off from the biological systems with higher genotoxicity and carcinogenicity (Madhavi and Salunkhe, 1995). It not only compromises human health but also due to its unknown fate, it may remain persistent in the environment leading to soil deteriorations putting forth the necessities of the biological ones (Liu and Mabury, 2020).

Antioxidants garnered the field of anticancer drugs by effectively scavenging the reactive oxygen species which when not given attention easily breaks up the nucleotides and thus the DNA strands (Manda et al., 2009). If it fails to repair on its own, it may alter the behavior and gene expression leading to uncontrollable cell-cycle regulations forming tumors and benign (Malumbres and Carnero, 2003). The drug development for cancer embraces the search for natural metabolites, since many herbals we are traditionally using are reported to be highly therapeutic, one such is the citrus family (Singh et al., 2020c). Citrus crops are documented for the major phytochemicals dealing the anticancer and antimicrobial actions (Singh et al., 2020b). Another important field that needs attention is that of multidrug resistance especially for the patients fighting cancer as major therapies leading the patients as immunocompromised individuals (Catalano et al., 2022). This expands the range of untreatable illnesses necessitating the urgency to seek out novel drugs to prevent the pathogenic infestations (Smolinski et al., 2003). The natural, safe bactericides/ fungicides that can be able to control infections and without complicating or interfering the cancer therapies are given attention (Mishra and Tiwari, 2011). In this light, the citrus possesses many phytochemicals which may equally be found to be antimicrobial besides their antioxidant potentials, and being used in culinary for centuries, the citrus is found to be very safe. However, the appropriate components responsible for these activities have not been reported in detail and was given a less attention. So, in this backdrop, this current study focussed to evaluate the various active components present in the methanolic extracts of the plant leaves by using the GC-MS analysis, evaluating the phytochemicals and antioxidants of this leaf extracts and to validate their antimicrobial potentials.

2. MATERIALS AND METHODS

The present analysis is on the vegetative parts of the plant *Citrus maximus,* mainly the leaves. Analytical grade chemicals were only employed in our study and were consumed appropriately.

2.1 Plant Collection:
The fresh leaves of *Citrus maxima* were collected from their natural habitat. Gudalur. The collected samples were identified and authenticated by Dr M.U Sharief, Scientist 'F', the plant identification, Department Botanical Survey of India, Coimbatore.

2.2 Preparation of plant extract
The powdered plant samples (10g/100ml) were extracted successively with methanol and water using an orbital shaker at 40°C for 24hrs with 60-70rpm. The corresponding extracted solvent was then collected and stored under 4°C until further use. After incubation, the extract was filtered and subjected to analysis for *in vitro* activities (Doughari, 2006).

2.3 PHYTOCHEMICAL ANALYSIS
The invitro phytochemicals of the plant leaf extracts (water and methanol) were assessed according to standard protocols by Tiwari et al., (Tiwari et al., 2011) and Rajesh et al., (Rajesh et al., 2014).

2.3.1 Test for alkaloids
Meyer's test was performed for the detection of alkaloids in the extract, in short, 1ml of the extract was added with 1ml of Mayor's reagent and observed for the colored precipitation. The formation of yellow colour precipitate indicates positive for the alkaloids.

2.3.2 Test for Terpenoids
For testing the Terpenoids, 1ml of the extract was taken added with 1ml of concentrated sulphuric acid and mixed well followed by heating for 2 minutes. The presence of the terpenoids is indicated by the formation of greyish colour.

2.3.3 Test for Phenols
Ferric chloride test was employed for the phenol detections. To test, a few drops of 10% ferric chloride solution was added to 1ml of the plant extract and kept observed for the appearance of blue-green/black coloration.

2.3.4 Test for Sugar (carbohydrates)
The Fehling's test was performed to evaluate the presence of the reducing sugar. 1ml of the extract was hydrolyzed with 70% of HCl and neutralized with an alkali before adding 1ml of each fehling's A and B solutions. It was then heated for 2 minutes to observe color changes if any. A red color formation indicated the presence of sugar.

2.3.5 Test for Saponins
Foam test was performed to check the saponins in the plant extracts. In a test tube, 1ml of the extract was taken and mixed with 1-2ml of distilled water and shaken vigorously. The formation of foam layer (atleast 1cm) indicates a positive result.

2.3.6 Test for Flavanoids
For testing the Flavanoids, 1ml of the extract was treated with a few fragments of magnesium and concentrated hydrochloric acid (a few drops). The presence of flavonoids is indicated by the appearance of pink, scarlet colour in a few minutes.

2.3.7 Test for Quinines
To 1ml of the extract, 2% of 1ml of sodium hydroxide was mixed and observed for the change in color. The presence of quinines is indicated by the appearance of blue green/ red colour.

2.3.8 Test for Proteins
To 1ml of the plant extract, few drops of mercuric chloride and nitric acid is added and observed for the formation of yellow colour.

2.3.9 Test for Steroids

The presence of Steroids was tested by adding 1ml chloroform and 1ml of concentrated sulphuric acid to 1ml of the extract taken in a test tube. The formation of red colour in the chloroform layer (lower layer) shows the presence of steroids (Evans, 2009; Harborne, 1998).

2.4 ANTIOXIDANT ACTIVITY

2.4.1 Total phenol:

The Folin-Coicalteu method was employed for phenol estimation. Shortly, 1ml of the leaf extract was mixed well with 0.5ml of 10% Folin-ciocalteu reagent and 2ml of the 20% Na_2CO_3 solution and kept shaking for 15 minutes at 45℃. Then it was read at 765nm using a spectrometer. The standard used was the gallic acid and the total phenol was expressed in mg/g (Senguttuvan et al., 2014).

2.4.2 DPPH

The free radical scavenging activity was assayed using DPPH. The DPPH solution (0.004%, w/v) was prepared in methanol and used for preparing stock solution (1 mg/ml). Abscorbic acid (0.05 g/ml) in methanol was used as standard. For testing, to 500µl of the leaf extract, 1ml of DPPH and 0.4 ml of 50mM tris HCl buffer and further incubated at dark for 30mins before reading at 517nm using a spectrophotometer (LT 291 labtronics microprocessor) (Blois, 1958). For blank, the methanol has been used and the DPPH activity was calculated using the formula:

Scavenging activity percentage= [(Ac−As)/Ac]×100

where: Ac = Absorbance of control, As = Absorbance of sample.

2.4.3. FRAP

The potassium ferricyanide-ferric chloride method was employed for estimating the ferric reducing capacity of the leaf extract. Briefly to 1 ml of the extract, 0.1M phosphate buffer solution and 2ml of 0.1% potassium ferricyanide and incubated at 50°C for 30 mins before adding 2 ml of the 10% trichloro acetic acid as stopping solution. Further it was centrifuged at 5000rpm for 5 mins and to the clear layer, 2ml of 0.1% FeCl3 solution was added and measured at 700nm using a spectrophotometer (LT 291 labtronics microprocessor). The results were expressed as mg/g of the FRAP by using ascorbic acid as standards (Chu et al., 2000; Yıldırım et al., 2001).

2.4.4. SOD

The superoxide dismutase activity was assessed based on their inhibition in photoreduction of nitrobluetetrazolium. Briefly, 0.1ml of leaf extract, 1 ml of mixture I (1ml of 50mM PBS, 0.075ml of 20mM L Methionine, 0.04ml of 10mM hydroxyl amine hydrochloride, and 0.1ml of 50mM EDTA) was mixed well and incubated 5mins at 30℃. To the incubated sample, riboflavin (50µM) was added and allowed to expose under fluorescence light (200W). Later, 1ml of the reaction mixture II (1% Sulphanilamide in 5% phosphoric acid) was added and read at the 543nm (Das et al., 2000). The percentage of inhibition was calculated as follows:

$$\% \text{ inhibition of nitrate formation} = 1 - \frac{AS}{AC} \text{ X } 100$$

where: Ac = Absorbance of control, As = Absorbance of sample.

2.4.5. Total antioxidant activity

Total antioxidants were assessed using the phosphor molybdenum methods by Phatakand Hendre (Phatak and Hendre, 2014) and Prieto et al (Prieto et al., 1999) with minor modification. Briefly, 0.5ml of the sample was mixed well with the 0.5ml of reaction mixture (0.6 M H2SO4, 28mM sodium phosphate and 4mM ammonium molybdate reagent) and kept incubated at 50℃ for 90 mins. Then the reading was taken at 695nm once samples normalized to room temperature. The ascorbic acid standard were used and the results

expressed in mg/gm of total antioxidant activity (Govindarajan et al., 2003; Mahdi-Pour et al., 2012).

2.5 CRUDE COMPOUND ANALYSIS

2.5.1. UV-Visible study

The methanol and water extract of the plant sample was examined under UV Visible spectral analysis. 2ml of the plant extracts were subjected to scanning in the UV-Vis spectrophotometer for the wavelength range of 200-800nm. The corresponding methanol and water were used as blanks (Easmin et al., 2017).

2.5.2. FTIR

The major functional groups present in the corresponding leaf extracts were found out by submitting the filtered extracts in FTIR-ATR equipment (Shimadzu instrument). All reading was performed in room conditions. The spectra were taken in the range of 4000–400cm^{-1} from the data presence of functional group was identified (Govindarajan et al., 2003).

2.5.3. TLC

TLC method was used for the identification of secondary metabolites. The chromatographic sheet was set with 5cm width and 8cm length and spotted with the sample 1cm above from the bottom using capillary tubes (about 50 µl). Sample placed and this was run in solvent system of methanol, acetic acid, formic acid and water, (6:1.8:1.8:1). By leaving 1cm from the top of the plate this was taken out from the solvent and dried to visualize the compound, the same method was fused for the TLC study (Ahamed et al., 2017). Rf value was calculated after visualization of the compound as spot in plate. Rf value calculation formula is;

$$\text{Rf value} = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

2.5.4. GC-MS

The GC-MS of the methanolic extract was assessed using the standard methods by Semwal and Painuli (Semwal and Painuli, 2019) with minor modifications. Shortly, the GC-MS system with the mas hunter method path (CH-GCMSMS, Agilent, United States). The oven temperature was set initially for 50°C and slowly set upto 280°C. In the column, 1ml of the extract (approx. 1 mg/ml) was injected and carrier gas employed was Helium (purity 99.99%: 1.2 ml/min). The resultant unique peaks were detected using a MS detector based on the different retention times and mass coverages. The peak areas were quantitatively determined for the determination of different compounds and cross checked with the standard library containing the different mass spectra. The complete running time of GC-MS lasted up to 33 min. The individual constituents and relative percentages were identified with the peak area normalization.

2.6 ANTIMICROBIAL ACTIVITY

The antimicrobial and antifungal activities of the leaf extracts were assessed using an agar well diffusion method. 70µl of fresh bacterial and fungal culture was pipetted in the centre of sterile (sterilization at 121°C for 15 minutes) Petri dishes (39g/L of Mueller Hinton agar and 38g/L of malt agar) containing solidified media. Followed by spread with cotton swabs and, wells were made using a sterile cork borer (6 mm in diameter). Then, 50µl of each extract was added to respective wells and the plates were incubated at 37°C for 24 hrs. The zone of inhibition around the wells was measured excluding the well diameter to found out the antimicrobial activity after incubation time. Water and methanol were employed as negative control and Gatifloxacin disc (GAT-5mcg against bacteria) and fluconazole (F-10mg/ml against fungi) were used as a positive control (Daoud et al., 2019; Gonelimali et al., 2018).

3. RESULTS AND DISCUSSION

The plants are primitive food sources and were consumed for centuries for curing various human diseases, collectively called medicinal plants. Many commercially available active molecules of the drug are inspired from these natural sources. Natural product chemistry led us to treat various diseases and disorders including common pathogenic infection to rare tumor disorders. One of the significances in opting the natural molecules from the plants is their lower cost and easier availabilities devoid of chemical synthetic requirements which mostly suits the populations of the low-income countries. *Citrus* is a widely grown variety that gained attention due to its multiple health benefits with refreshing flavor. The metabolites of *Citrus maxima* leaves were extracted with water and methanol and analyzed further for evaluating their phytochemicals and antimicrobial activities. The results showed the presence of various secondary metabolites including alkanoids, terpenoids, phenols, saponins, flavonoids, quinines and steroids besides the proteins and carbohydrates (Table. 1). Methanol was found to extract the maximum metabolites when compared to the water medium (Sapkota et al.).

(Insert Table.1 here)

The phytochemicals of a plant including saponin, phenols and alkaloids are strongly linked to render for their pharmacological effects. The phytochemicals including the terpenoids, phenols and flavonoids are well extracted using both the solvent systems. The *Citrus sp*. is found to be rich in polyphenols with more than 60 different flavonoids coming under six types: flavones, isoflavones, catechins, flavanones, anthocyanidins, and flavonols (Zahr et al., 2023). A similar report by Federica Menichini., (Menichini et al., 2011) documented that the presence of various bioactive compounds in the *Citrus* fruits are responsible for their beneficial effects, especially the flavonoids. There are many different types of flavonoids present in the Citrus as per records that includes naringeninm hesperetin, rutin, nobiletin, naringenin, tangertin, apigenin, diosmin and quercetin. Whereas some of the phytochemicals including the alkaloids, saponins and quinines were extracted well either with water or with methanol solvent systems. This must be due to the differences in the efficacy of the solvent systems based on their concentration that have been employed for the extraction. Our results are in alignment with the findings by J.M. Ehiobu, (Ehiobu et al., 2021) who documented the varied extraction potentials of water and methanol employed in extracting the phytochemicals from leaf of *C. limon*.

3.2 Antioxidant activity

The free radicals like ROS are generally produced during the oxidation reactions which severely damage the macromolecules including lipids, proteins, DNA and RNA causing many degenerative disorders. Thereby the prevention of such molecules is mandated to overcome the undesirable effects, one such may be contributed by the action of antioxidants that can proficiently scavenges these (Singh et al., 2020a). Phytochemicals of a plant system is acknowledged for their bioactivities including the antioxidative and anti-inflammatory effects besides their antagonistic potentials which are majorly guided by their redox potentials playing prime role in scavenging the free radicals generated in a system (Zheng and Wang, 2001). A report said, there were more than 170 antioxidants reported in Citrus fruits (including various phenolics, vitamins, pectins, terpenoids and other mineral elements) (Li et al., 2022).

The Antioxidant activity was performed using TPC (Total phenol Content), DPPH Assay, FRAP, SOD and total antioxidant activity were determined (Table.2). The Folin–Ciocalteu's assay is a prominent method of measuring the total phenolics in the medicinal plants (Khettal

et al., 2017). The values of this study reside in the presence of very strong natural Antioxidant in the aqueous and methanol extract leaves of the *Citrus maxima*. The TPC in the aqueous extract is 154mg/g of total phenol and in methanol extract is 130mg/g of total phenol. In a similar study by Bachra Khettal, (Khettal et al., 2017), the results were different from ours, they reported that the total phenolics in various citrus leaves (*C. clementina*, *C. limon*, *C. hamlin*, *C. navel*, *C. aurantifolia*, *C. aurantium* and *C. grandis*) were found to be higher in case of methanolic extract (2.4 to 11.6 mg/g) than that of the aqueous extract (68.2-125.2 mg.g).

In general, the DPPH assay validates the radical scavenging activity in an inhibitory percentage. The percentage of the DPPH scavenging activity is 21.6 in aqueous and 34.6 in methanol extract. Antioxidants are documented to intercept the free oxidants to donate the hydrogen from the phenolic hydroxyl group to become stable (Yehye et al., 2015). FRAP content in methanol extract is very high and the value is in triplets (141mg/g) when compared to aqueous extracts. In a study by Changjiang Guo, (Guo et al., 2003) reported different FRAP values for the peel, pulp and seeds of fruits, their results showed that a total of 2.23 mmol/100g wet weight of pomelo fruit. Superoxide dismutase is a major antioxidant in the enzymatic antioxidant system in plant systems. Our results revealed the SOD in the range of 13 and 33 in water and methanol extracts respectively. An ion containing cyanide insensitive SOD was purified from *Citrus limonum R* leaves and its specificity was found to be 1.500 U/mg (Almansa et al., 1991). The total antioxidants were found to be 710 in aqueous extract and a little less around 606 in methanolic extracts. Total antioxidant activity shows that the citrus leaf contains strong antioxidants which are important to prevent oxidation (Khan et al., 2018).

(Insert Table.2 here)

3.3 Compound Analysis

The major compounds present in the plant sample were studied using the UV-Visible study, FTIR (Fourier-transform infrared spectroscopy), TLC and GC-MS**.** The compound identified by this study shows various biological activities like antimicrobial, antioxidant, antifungal, and anti-inflammatory (Lukitaningsih and Rumiyati, 2021).

3.3.1 UV-Visible study

The UV-Vis spectroscopy leads to understanding the UV radiation absorption through the excitation of electrons in each molecule to a greater state of energy. The given are the results of the UV-Vis spectrum for the leaf extracts (both aqueous and methanolic) and standard rutin. All the samples showed three distinct peaks in the absorbance range of 325 to 345 (Fig. 1, a-c). The methanolic extracts showed a prominent peak at 665nm (Fig.1a) which was found to be absent from the aqueous leaf extracts (Fig. 1b). The UV- spectrum of standard Rutin is given in Fig.1c. As is well known the extraction potential of the components differed when employing different solvents, few compounds may be extracted well in methanol rather than other solvent systems.

(Insert Fig1. Here)

3.3.2 TLC

The screening and preliminary analysis of different molecules in a compound or a natural extract is the first step while examining biological activity prior proceeding with any in vivo studies. Besides various chromatographic techniques, the Thin Layer Chromatography is specially preferred for screening experimentation to know more about the various component present and their separation and efficiency of the solvent systems assisting such separations. Another important feature is that many samples may even be compared by spotting side by side which is impossible to perform in any other chromatographic techniques (Łukasz M.

Cieśla , 2015). Our results revealed a clear separation with the solvent system of methanol, acetic acid, formic acid and water. The TLC profile was obtained against the rutin standard (0.641) and the Rf value obtained with the solvents water is 0.679 and methanol is 0.634.

(Insert Table.3 here)

3.3.3. FTIR

The FTIR analysis showed a similar spectrum for both water and methanolic extracts of the Citrus maxima leaves (Fig. 2). The results indicated the predominant vibration of stretching and bending according to the corresponding molecules absorption. The major functional groups found were given in table.4. There existed a alcohol stretching vibration in the wavelength ranges of 3325.28 cm^{-1} (Jabamalairaj et al., 2015) and 1018.41 cm^{-1} and alkanes at 2831.50cm^{-1} and 2947.23cm^{-1}. The presence of aromatics was confirmed by the stretching vibration at absorption wavelength of 1450.47cm^{-1}. The presence of carbonyl group was observed at 1111cm^{-1}.

(Insert Table. 4 here)

(Insert Fig.2 here)

3.3.4. GC MS Analysis

A total of 15 compounds were identified from the GC-MS analysis of the methanol fraction of *Citrus maxima* leaf extract exhibiting various phytochemical activities. The Chromatogram is presented in Fig. 3, and the molecular formula and the biological function of various compounds of the identified compounds are given in the table (Table.5). Among the identified compounds by GC-MS analysis the compounds 1,2,3,8,9,10, 11 and 14 exhibit the anticancer, antimicrobial and anti-inflammatory activity. The compounds 6,7 exhibit antioxidant and anti-inflammatory activity. Compounds 5, 12, and 13 exhibit antibacterial activity. Compound 4 exhibit Antiplasmodial and compound 15 exhibit anti leishmanial activity.

(Insert Fig.3 here)

(Insert Table 5 here)

3.4 Antagonistic activity:

Pathogenic bacteria and fungi are the major cause for the increasing mortality rate in many countries with the data shown of about 50,000 people dying every day due to multiple infections (Asker et al., 2020). Many phytochemicals are reported to efficiently control the growth of various diseases causing virulent bacterial and fungal strains. The main mechanisms behind this may include the rupturing of cell walls leading to the cellular damages, metabolic dysfunction, inhibition of protein or nucleic acid synthesis, cell pyknosis and eventually the cell death (Liang et al., 2023). Further, these pathogens are getting virulent day by day by becoming antibiotic resistant against different drugs used in healthcare sectors which calls for the phytochemical research to come up with efficient than existing drugs. The antibacterial and antifungal activities of the leaf extract of the *Citrus maxima* are performed by the agar well diffusion method. The antibacterial activity of the plant extract shows the zone of inhibition and is measured in mm. *P. aeruginosa, E. coli, B. subtilis and S. aureus* are the four bacteria used in this study. A zone of inhibition was noticed against all the four bacteria (Table.6). Thus, aqueous extract of the plant samples shows strong antibacterial properties. The present study of the plant extract also shows the antifungal properties against two fungi *A.niger and A.flavus.* These results were similar with another study by Lukitaningsih (Lukitaningsih and Rumiyati, 2021).

(Insert Table.6 here)

Conclusion:

One of the important plants of citrus family, the *C. maxima* found to offer a wide range of ethanomedicinal and nutritional benefits. Almost all the plant parts especially the leaves are the cure for many diseases and disorders. The phytochemical profilin of this plant leaves extract showed the present of alkaloids, phenols, flavonoids, terpenoids, saponins, quinines, steroids, proteins and carbohydrates. These extracts exhibited various pharmacological properties including antioxidant potential through their potential DDPH, FRAP, SOD assays and were found to show potent antagonism against various common bacterial and fungal human pathogens. An attempt were also made to document the various phytochemicals present in the leaf extracts through characterization including TLC, UV-Vis spectroscopy, FTIR and also GC-MS analysis. The GC-MS analysis showed the presence of various chemical components responsible for antimicrobial, anti-inflammatory, antioxidant, antitumor and anticancer properties. Further studies will be carried out to check out these potential properties *in vivo* using animal models so that it may open the doors for drug formulation to combat the cancer and to treat the pathogenesis in immune compromised individuals.

Statements and declarations:

The authors declare that there are no conflicts of interest and all authors agreed and approved for submission.

Authorship credits:

Priya: Conceptualization, experimentations and drafting
Parimala: Drafting, Review, Editing, and Supervision
Minisha: Figures, tables preparation
Ayyappa Das: Conceptualization, supervision and Editing

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Fig. 1 UV-Vis spectrum for samples and Standard

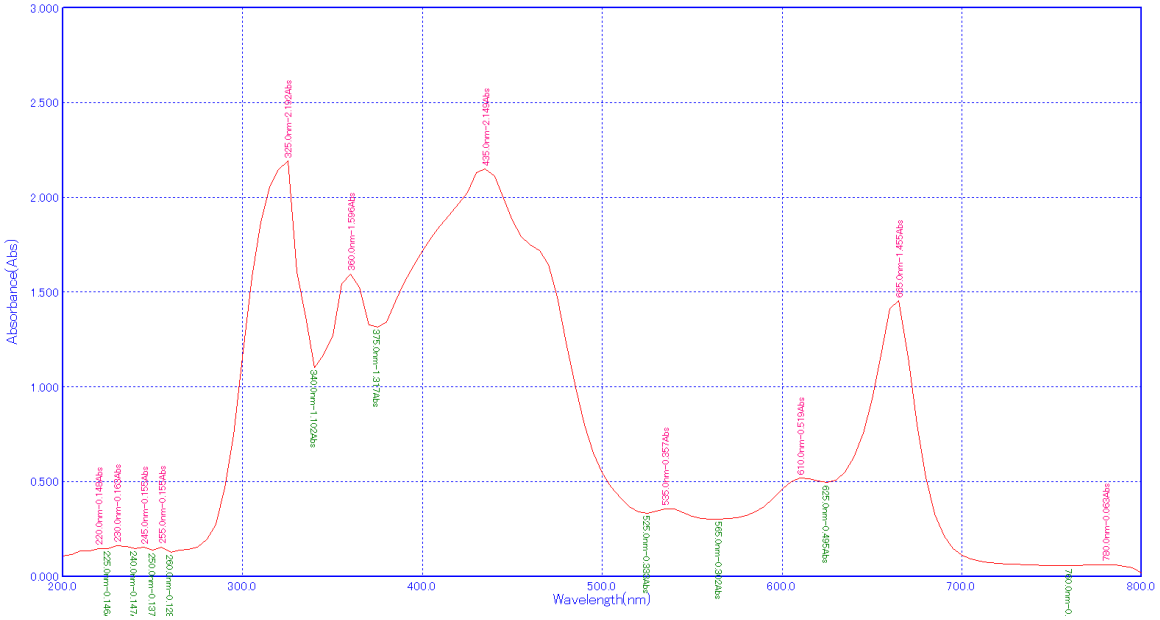


Fig.1a Uv-Vis spectrum of Methanolic leaf extract of *Citrus maximus*

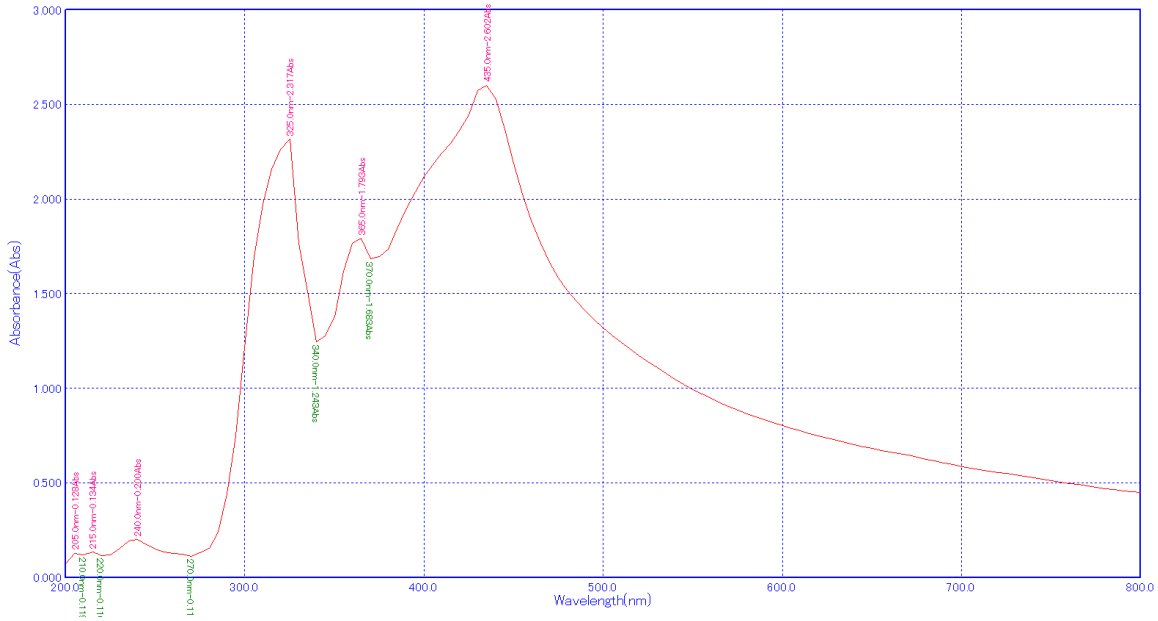


Fig.1b Uv-Vis spectrum of Aqueous leaf extract of *Citrus maximus*

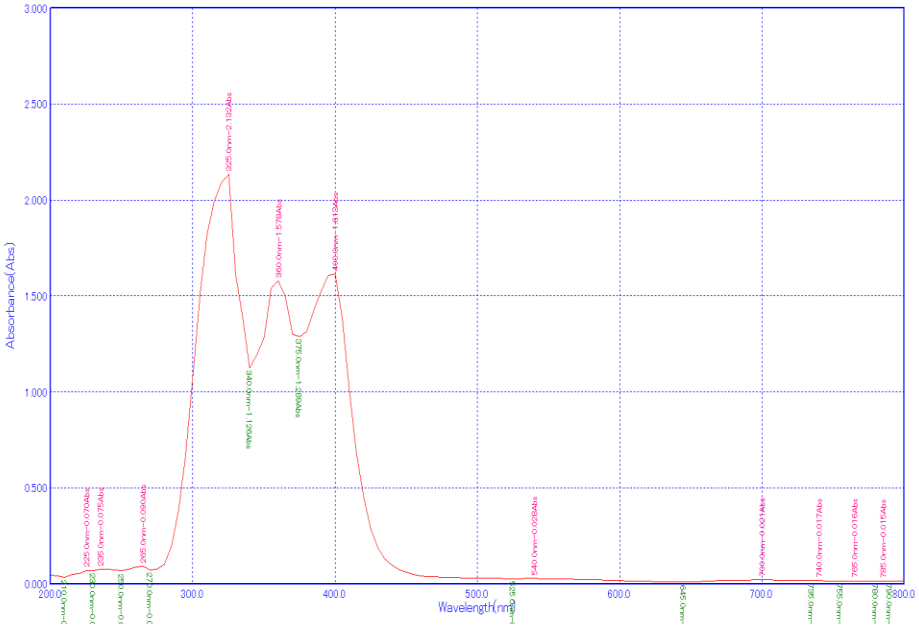
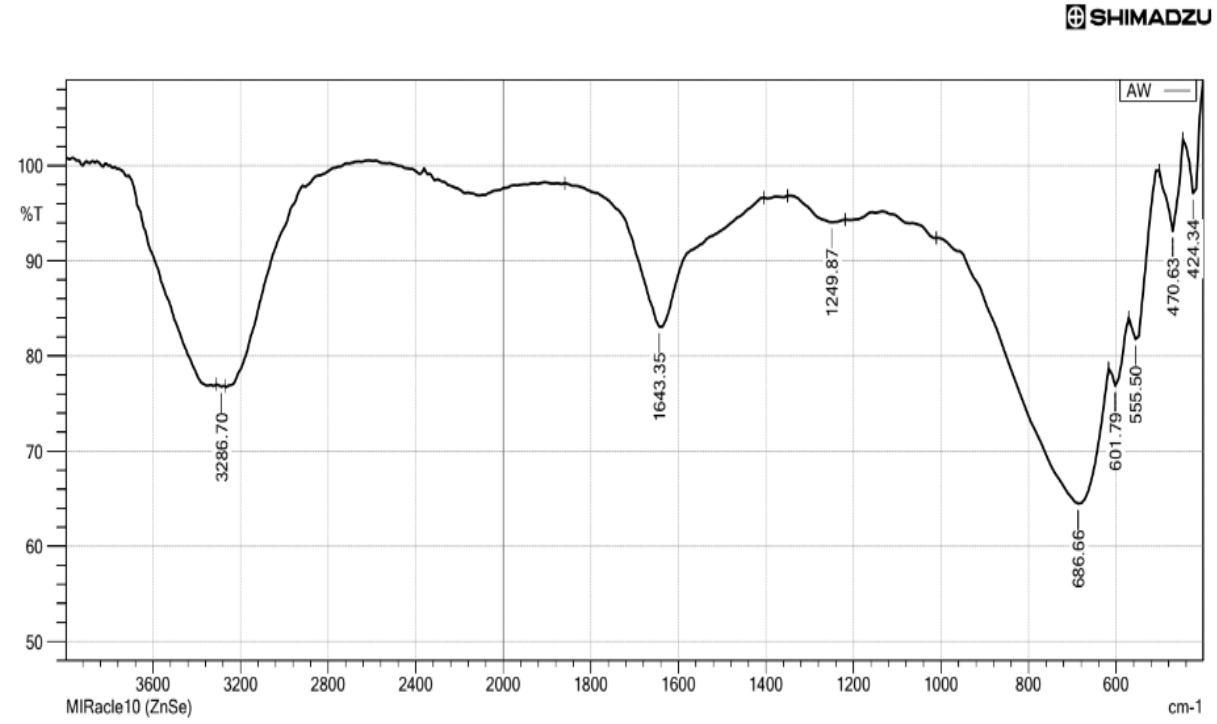


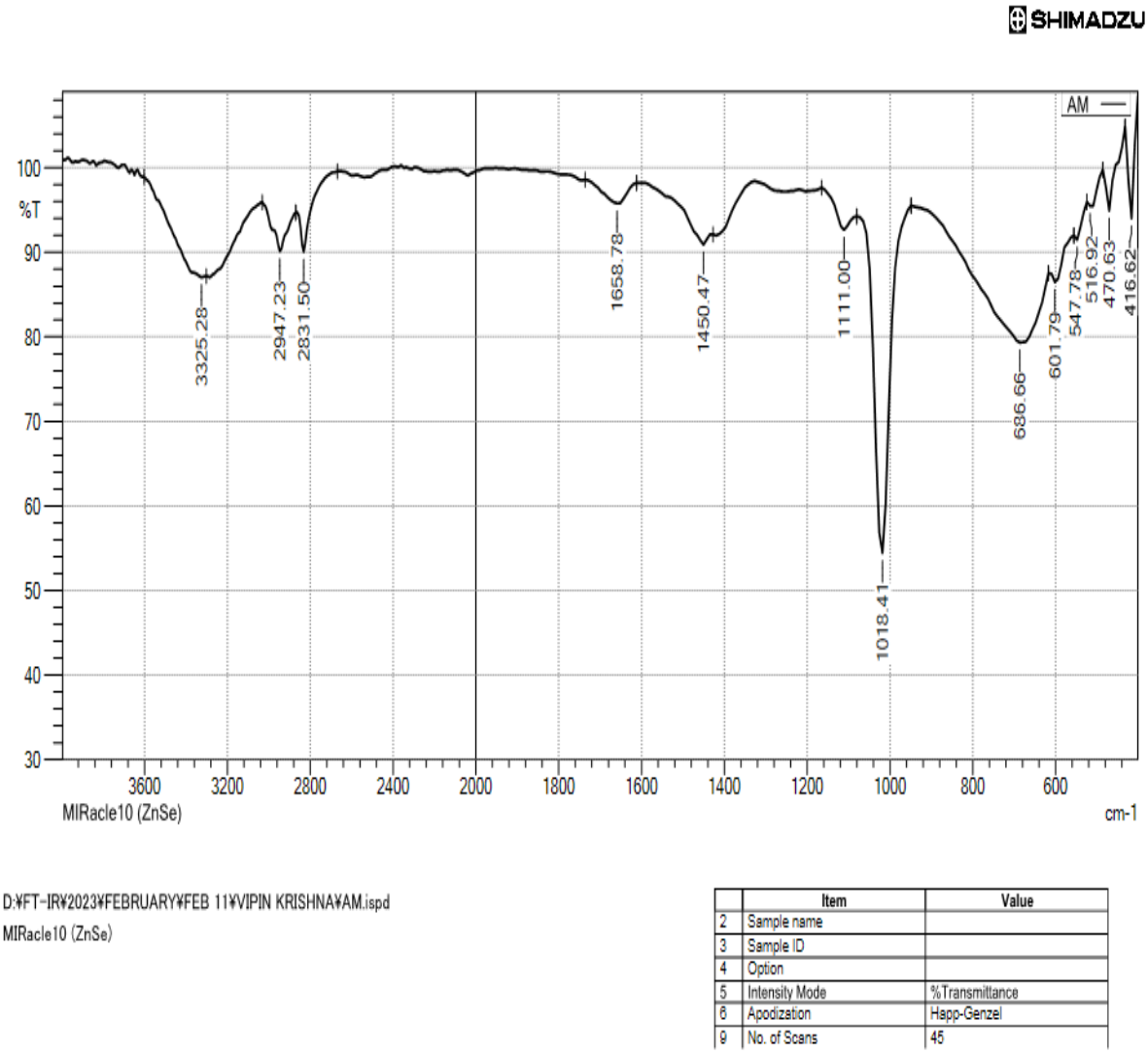
Fig.1c. Standard – Rutin sample (in Ethanol)



D:¥FT-IR¥2023¥FEBRUARY¥FEB 11¥VIPIN KRISHNA¥AW.ispd
MIRacle10 (ZnSe)

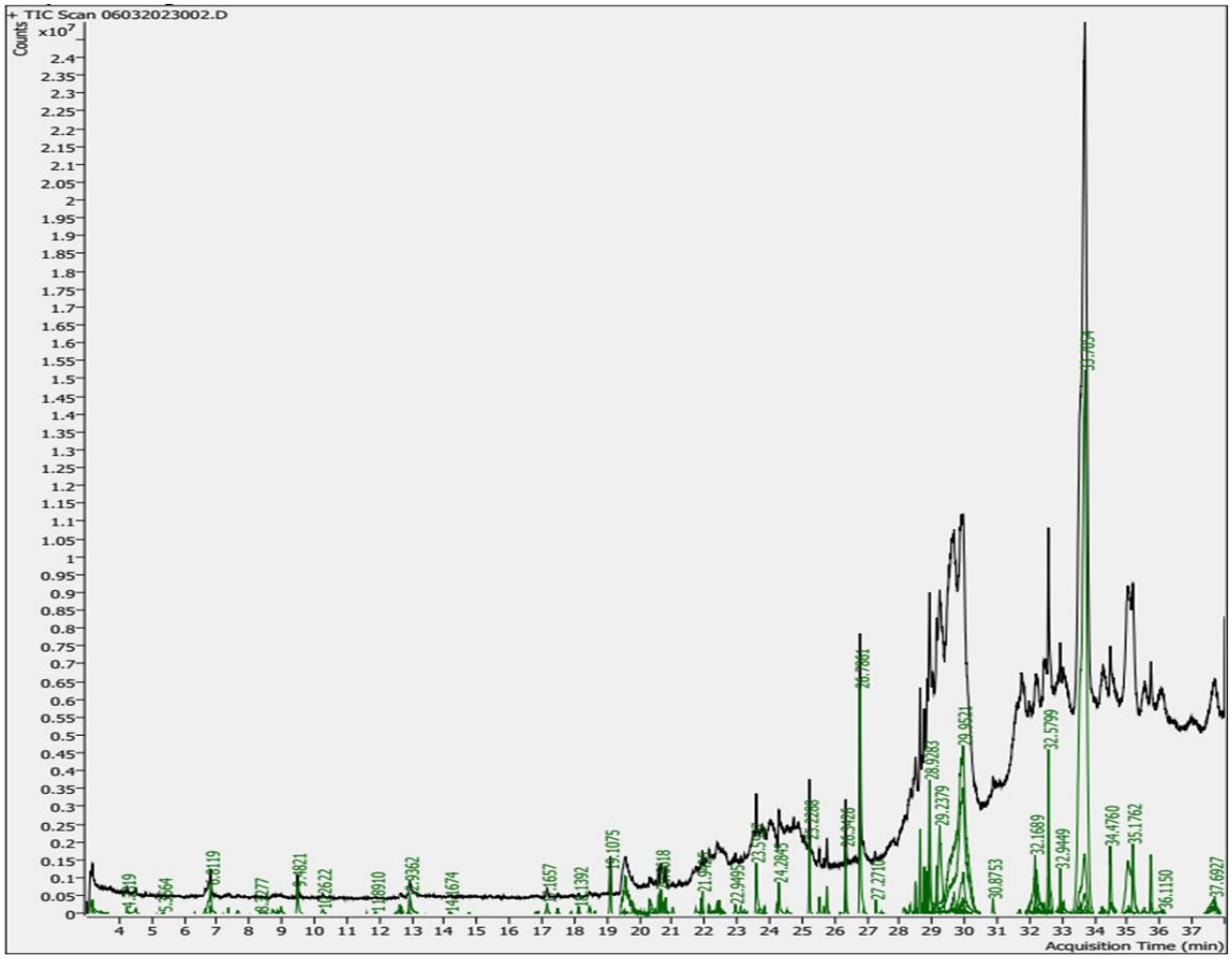
	Item	Value
2	Sample name	
3	Sample ID	
4	Option	
5	Intensity Mode	%Transmittance
6	Apodization	Happ-Genzel
9	No. of Scans	45

Fig.2. FTIR spectrum of aqueous &methanolic leaf extract



	Item	Value
2	Sample name	
3	Sample ID	
4	Option	
5	Intensity Mode	%Transmittance
6	Apodization	Happ-Genzel
9	No. of Scans	45

Fig 3. GC-MS spectrum of methanolic leaf extract



Tables

Phytochemical constituents	*Citrus maxima* leaf extract	
	Aqueous	**Methanol**
Alkaloids	-	+
Terpenoids	+	+
Phenols	+	+
Sugar	-	+
Saponins	+	-
Flavanoids	+	+
Quinines	-	+
Proteins	+	-
Steroids	-	+

+ indicated presence and -indicated absence

Table.1: Various phytochemical constituents of Citrus maxima leaf extracts using water and methanol

Table. 2 The antioxidants in the aqueous an methanolic leaf extract of *Citrus maximus*

Sample/Assay	Phenol	DPPH	FRAP	SOD	Total antioxidant
Aqueous Extract	154±0.94	21.6±0.23	56.2±0.2	12.9±0.2	710±1.24
Methanol Extract	130±0.19	34.6±1.7	141±1.2	33.21±1.2	606±0.81

Table. 3. TLC for leaf extracts of *Citrus maximus*

Citrus leaf extract	**Rf value**
Water	0.679
Methanol	0.634
Standard (Rutin)	0.641

Table. 4. Functional groups and characteristics absorption wavelength of *Citrus maxima* leaf extract

Functional groups	Types of vibrations	Characteristic absorption (cm^{-1})	
		Aqueous Extract	Methanolic Extract
Alcohol	O-H (stretch) C-O (stretch)	3325.28 1018.41	3286.70 -
Alkanes	-C-H (bend) C-H (stretch) (rock)	2831.50 2947.23	- 1249.87
Alkenes	C=C (stretch)	1658.78	1643.35
Aromatics	C=C (stretch)	1450.47	1249.87
Carbonyl	C=O (stretch)	1111.00	-
Halide	C-Br	686.66	686.66

Table.5. The GC-MS predicted bioactive components of *Citrus maxima* methanolic leaf extract

S.no	**Name of the compound**	**Molecular formula**	**Biological function**
1	Neophytadiene	C20H38	Anticancer and Antimicrobial (Al-Rajhi et al., 2022)
2	Ursolic aldehyde	C30H48O2	Anti-inflammatory and Anticancer (Dar et al., 2016)
3	Thiophene	C5H6S	Antimicrobial (Liao et al., 2018)
4	Indole	C14H11N3O2	Antiplasmodial activity (Frederich et al., 2008)
5	3-Quinolinecarboxylic acid, 6,8-difluoro-4-hydroxy-, ethyl ester	C12H9F2NO3	Antibacterial (Wentland et al., 1984)
6	Phloroglucinaldehyde	C16H30O4Si3	Antioxidant (Sivamaruthi et al., 2016)
7	Elemene	C15H24	Anti-inflammatory, antioxidant. (Chen et al., 2023)
8	Phenol, 3,5-bis(1,1-dimethylethyl)	C14H22O	Anti-inflammatory and antimicrobial activity and Anti Cancer (Tripoli et al., 2005)

9	1-Tetradecanamine, N,N-dimethyl	C16H35N	Antibacterial, antifungal, anti-cancer (Kalaba et al., 2022)
10	Phytol	C20H40O	Cytotoxic, antioxidant, anti-inflammatory, antimicrobial (Gliszczyńska et al., 2021; Islam et al., 2018)
11	Octadecanoic acid	C18H36O2	Antifungal, antitumor activity, antibacterial (Akpuaka et al., 2013)
12	Piperonal	C8H6O3	Antibacterial (Beckford et al., 2011)
13	Fumaric acid, ethyl 3-nitrophenyl ester	C12H11NO6	Antibacterial (He et al., 2011)
14	Supraene	C30H50	Antimicrobial (SE et al., 2018)
15	Docosenamide	C22H43NO	Anti leishmanial (Gololo et al., 2016)

Table 6. Antagonistic potentials of *Citrus maxima* leaf extracts

Name of the Pathogen	**Zone of inhibition in mm**				
	WE	ME	Disc (antibiotics)	W	M
P aeruginosa	7	5	6	Nil	Nil
E. coli	5	8	7	Nil	Nil
B. subtilis	6	7	7	Nil	Nil
S. aureus	6	7	9	Nil	Nil
A.niger	1	3	5	Nil	Nil
A.flavus	3	5	6	Nil	Nil

*W- Distilled water; M- methanol; WE-water extract; ME-Methanolic extract; Disc - antibiotic

Date: 15.06.2024
To
The Editor-in-chief
African journal of biological sciences

Respected Prof.,
Sub: Submission of manuscript for publication in your esteemed Journal- Reg.,
I hereby submit the manuscript entitled "**Comprehensive Phytochemical Profiling and Biological Activity Assessment of Citrus maxima Leaf Extracts: Insights from GC-MS Analysis**" for consideration to be published under the esteemed journal "**African journal of biological science**".

I assure you that this manuscript has neither been published in any other journal nor submitted for publication in any other journal. I also undertake along with the other authors that animal/human study was taken after the prior approval of relevant country/institutional ethical committee. I and on behalf of other co- author, declare "No conflict of interest". Kindly consider the manuscript for publication in your journal. I abide by all rules and regulations of the journal. In future if any litigation arises in this article, I will cooperate with the editor to resolve the issue. I accept the decision of the editor would be final.