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PHYTOCHEMICAL PROFILE AND *IN VITRO* ANTIOXIDANT, ANTI-BACTERIAL AND ANTI-INFLAMMATORY POTENTIAL OF *PIPER WIGHTII* MIQ: A WILD MEDICINAL PLANT

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ABSTRACT

Piper wightii a well-known medicinal plant of Piperaceae family is used by many tribal groups to treat inflammation in India. In the study, different extracts such as petroleum ether, chloroform, ethyl acetate, ethanol and hot water of *Piper wightii* leaves are evaluated for its antioxidant, antibacterial and anti-inflammatory activity and phyto constituents are profiled using Gas Chromatography – Mass Spectrometry (GC-MS). Among various extracts, leaf ethanol extracts showed the maximum amount of phenolics (336.84mg GAE/g extract), tannin (331.53mg GAE/g extract) and flavonoids ethyl acetate (174.88mg RE/g extract) content. It also revealed the presence of highest antioxidant property by estimating DPPH% (IC_{50}: 21.03µg/mL), ABTS+ (64166.67µM TE/g extract), Superoxide (44.84%) radical scavenging activity and Phosphomolybdenum ethyl acetate (177.25mg AAE/g). Moreover, ethanol extract exhibited good antibacterial activity against *Klebsiella pneumonia* (22.03mm), *Escherichia coli* (22.35mm), *Salmonella typhimurium* (25.23mm) *Bacillus subtilis* (22.3mm), *Staphylococcus aureus* (20.37mm), at 20mg/ml concentration. The ethanol extract showed high degree of inhibition (63.69%) in anti-inflammatory assay. GC-MS depicted the presence E, E, Z-1, 3, 12-Nonadecatriene-5, 14-diol component primarily in leaf ethanol extract. *Piper wightii* leaf extracts have a tremendous amount of antioxidant potential, making them a good source of natural antioxidant supplements for food to protect against oxidative stress-related diseases, including inflammation.

KEYWORDS

Antioxidant, Anti-bacterial, Anti-inflammatory and *Piper wightii.*

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INTRODUCTON

India has a rich source of medicinal plants and many plant-derived extracts are used in several medical systems, including Ayurveda, Unani and Siddha, to treat various ailments[1]. Most of the people use medicinal plants for illnesses but few have undergone scientific investigation[2]. The medicinal plants contain an inexpensive source of medicine, they produce a variety of chemical

compounds that can be employed as building blocks to create new medications with improved pharmacological effects[3].

Inflammatory diseases are treated worldwide by using synthetic drugs and many of them have serious side effects[4]. For example, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) is most commonly used for the inflammatory management and exhibit many adverse side effects[5]. Also, steroids and antihistamines play a major role in treating anti-inflammatory diseases, the prolonged intake of these drugs causes various diseases such as asthma, gastric ulcer, arthritis, cancer, rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, Crohn's disease, multiple sclerosis and systemic vacuities[6]. There is a wide search for novel therapeutic drugs without any side effects and the plant kingdom holds the novel compounds with significant anti-inflammatory activity[7].

The *Piper wightii* plants are the wild type of edible pepper commonly known as kattukurumilagu. They are mostly distributed in the evergreen Shola forests of the Western Ghats, south India and are endemic to Peninsular and North East India. The scientific evidence supporting the anti-inflammation activity of *Piper wightii* is lacking and scientific validations are needed to highlight the phytochemical and pharmacological properties of the indigenous wild endemic pepper varieties of the Nilgiris. Hence, the main objective of the present investigation is to evaluate *in vitro* antioxidant, antibacterial and anti-inflammatory properties.

MATERIAL AND METHODS

Plant collection and extract preparation

The fresh leaves of *Piper wightii* were collected from Coonoor on September, 2021 and they were certified by the Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamilnadu. The fresh leaves were cleaned, shade dried and powdered for the investigations. About 70g of powdered leaves were dissolved in 500ml of various solvents *viz*., petroleum ether, chloroform, ethyl acetate, ethanol and hot water and extractions were prepared using soxhlet apparatus. The extractions were dried and used for antioxidant analysis.

Quantification of bioactive secondary metabolites

Determination of Total Phenolic Contents

The total phenolic content of *P. wightii* leaves from different solvent extracts was determined by the standard UV spectrophotometric (725nm) method[8].

Determination of Tannin Contents

Polyvinyl polypyrrolidone (PVPP) was used to treat the plant extract and the Folin-Ciocalteu method was used to figure out how much tannin was in the extract. The test was done three times, and the results were given in terms of gallic acid equivalents. As shown in[8], the tannic acid equivalents (TAE) were used to figure out how much total phenolics and tannins there were.

Determination of Flavonoid Contents

The aluminium chloride colorimetric assay was used to measure the amount of flavonoids in the plant materials[9]. Samples were analysed three times, and flavonoids were measured in terms of how much rutin they were equivalent to (RE).

In vitro antioxidant activity

2, 2-diphenyl -picryl-1-picryl-hydrazyl radical (DPPH)

The DPPH radical scavenging method was used to measure the radical scavenging activity of different solvent extracts of *Piper wightii* leaves. This method is written as[10]. Estimates were made of the IC_{50} values of the extract or the amount of extract needed to cut the initial amount of DPPH by 50%.

Phosphomolybdenum Assay

Using the methods of[11], the formation of a green phosphomolybdenum complex was used to measure the antioxidant activity of the samples. The results were given in milligrammes of equivalent ascorbic acid per gramme of extract.

2, 2 Azinobis (3-ethyl-benzothiozoline-6-sulfonic acid) disodium salt (ABTS)

The ABTS radical cation decolorization assay was used to measure the total antioxidant activity of different solvent extracts of *Piper wightii* leaves, as described[12]. The results were given as the concentration of trolex-equivalent antioxidant

activity, which was given as s mol/g sample extracts.

Superoxide radical Scavenging Activity

The test was based on how well different extracts stopped the formation of formazan by getting rid of the superoxide radicals made by the riboflavin light NBT system[13]. The superoxide anion scavenging activity was calculated as follows: Scavenging activity (%) = [(Control OD – Sample OD)/Control OD] x 100.

Antibacterial Activity of Plants Extract

Bacterial strains

The Department of Biotechnology at Bharathiar University in Coimbatore provided the five strains of dangerous bacteria used in this study. The bacterial strains used were *Klebsiella pneumonia* (MTCC10309), *Escherichia coli* (MTCC405), *Bacillus subtilis* (MTCC2057), *Salmonella typhimurium* (MTCC3224) and *Staphylococcus aureus* (MTCC9760).

Disk Diffusion Method

The disc diffusion method is employed to appraise the antimicrobial activity of every plant extract. The plant extract residues (20mg) were dissolved in 1ml of DMSO. Muller Hinton agar was then prepared under sterile conditions. The organisms that were isolated were inoculated in the nutrient broth and incubated overnight. Then, swabs were used to transfer the organisms to the Muller Hinton agar plates and then, sterile discs were placed. The plant extract solution of 30µl were poured into the sterile discs. The plates are incubated at 37ºC overnight[14].

***In vitro* Anti-inflammatory Activity**

Membrane Stabilization Method

2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride were dissolved in distilled water to create Alsever's solution, which was then sterilised[15]. The amount of haemoglobin in the supernatant solution was estimated by spectrophotometry to be 560nm. The following formula was used to figure out how much activity there was in stabilising membranes:

$$\text{Percentage inhibition} = \frac{\text{Control} - \text{Treated sample}}{\text{Control}} \times 100$$

GC-MS Analysis

Preparation of extract

One 1µL of the ethanol extracts of *Piper wightii* leaves were employed for GC/MS analysis.

Instruments and chromatographic conditions

A GC clarus 500 Perkin Elmer system with an AOC-20i auto sampler, a gas chromatograph, and a mass spectrometer were used for the GC-MS analysis (GC-MS). The following circumstances were applied: column Elite-1 fused silica capillary column (300.25mm i.d. 1EM df, 100% dimethyl polysiloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as the carrier gas at a constant flow of 1mL/min; 0.5 EI was used as the injection volume (split ratio of 10:1); injector temperature 250^{o}C; ion-source The temperature of the oven was programmed to rise gradually from 110°C (with no change for 2 minutes) to 200°C/min, then from 5°C/min to 280°C/min, with no change at 280°C for 9 minutes. With a scan interval of 0.5 s, 70 eV and fragment sizes ranging from 40 to 550 Da, mass spectra were recorded. Parts identification: In the National Institute of Standard Technology (NIST) library, the mass spectra of recognised and unknown components were compared. We learned the parts of the test sample's names, molecular weights, and structures.

Statistical analyses

All of the tests were done three times, and the results were given as the average plus the standard deviation. For antioxidant studies, a one-way ANOVA and then Duncan's test were used to analyse the data statistically. When $p< 0.05$, the value of the mean was considered statistically significant.

RESULTS AND DISCUSSION

Quantification of secondary metabolites

Determination of total phenolic contents

Total phenolic content has the potential to benefit human health; polyphenols, which are frequently found in plants, have antimutagenic, anticancer, antioxidant and free radical scavenging properties[16]. Total phenolic content of *Piper wightii* was higher in ethanol extract (336.84mg GAE/g) compared to other extracts such as water (120.76mg GAE/),

ethyl acetate, (96.78mg GAE/g), petroleum ether (60.23mg GAE/g) and chloroform (59.35mg GAE/g) (Table No.1). A perfect structural chemistry for scavenging free radicals is attained by the polyphenols and the wide group of phenolic chemicals generated from plants. Due to their higher responsiveness to acting as hydrogen donor and their competent to stabilize and delocalize the lone paired electron (chain-breaking function), polyphenols have antioxidant properties[17].

Determination of tannin contents

The leaf material revealed that it has a high flavonoid content and the ethyl acetate extract retains the maximum (331.52 RE/100g), while the petroleum ether extract showed the least amount (59.16g QE/100g) (Table No.1). Every sample extract has polyphenolic content that has performed as the best electron and hydrogen atom donor and consequently it shows the effectiveness to cease the radical chain reaction by bringing around the free radicals to more steady products. It is eminent to examine the correspondence between the content of total polyphenols and their antioxidant capacity because some authors have given account of the lack of a parallel connection between the content of these main antioxidant compounds and their radial scavenging activity[18].

Determination of flavonoid contents

Flavonoids are the most common group of polyphenolic compounds and are found almost everywhere. These are important antioxidants because they get rid of free radicals and bind to trace elements[19,20]. The flavonoid contents are analysed in the leaf of *Piper wightii* and the consequent outcomes are projected in the Table No.1. An ethyl acetate leaf extract of *Piper wightii* (174.88mg RE/100g extract) was observed to have prominent flavonoid contents associated with the former solvent extracts.

***In vitro* antioxidant activity**

2, 2-diphenyl -picryl-1-picryl-hydrazyl radical (DPPH)

The DPPH radical scavenging activity of the plant extract of *Piper wightii* leaf was assessed by equating with standards such as rutin and BHT (Figure No.1). The amount of extract required to produce 50% of the radicals was estimated using the IC_{50} of several extracts, which is significant. A lower IC50 value denotes greater antioxidant activity. The outcome indicates the ethanol extract of *Piper wightii* exposed the highest activity (IC_{50} 21.03g/mL), followed by the water extract (IC_{50} 34.43g/mL). DPPH decreases and decolorizes due to the hydrogen-donating properties of cysteine, glutathione, ascorbic acid, tocopherol, flavonoids, tannins, and aromatic amines[21]. As a result, the data obtained can be shown that all *Piper wightii* leaf extracts contain potential deterrent compounds that may function as primary antioxidants that interact with DPPH radicals. In many plant species, there seems to be a strong connection between total phenolic and antioxidant activity[22].

Phosphomolybdenum assay

The phosphomolybdenum analysis was used to establish the total antioxidant competence of *Piper wightii* leaf and the results are exhibited in Figure No.2. Ethyl acetate extracts *Piper wightii* leaf confirmed that it had a very high reducing ability (177.25mg AAE/g extract) however the ethanol extracts might show off the important extent of reducing ability (152.3mg AAE/g extract) when compared to other extracts. A study on phosphomolybdenum reduction was conducted to examine the antioxidant molecule in the sample's proficiency to reduce Mo (VI) to Mo (V). Active extracts for *Piper wightii* showed this ability to reduce relatively effectively. They have the capability to diminish the effect produced by an effective electron donor present in this extract. Tests have provided unambiguous evidence of the link between an active extract's phenolic content and its antioxidant activity[23].

2, 2 Azinobis (3-ethyl-benzothiozoline-6-sulfonic acid) disodium salt (ABTS)

In the ABTS cation radical scavenging method, the action of extracts tested was explicit as the micromolar equivalent of Trolox solution having an antioxidant correspondent to the dry matter of one gramme of the sample under the experimental exploration. To carry out the various solvent extracts of *Piper wightii* see Table No.2. Although the samples exhibit the auspicious ABTS radical

scavenging activity, the ethanol exhibited the greatest activity (64166.67µM TE/g extract) and abide by ethyl acetate (58298.61µM TE/g extract). The standard natural antioxidant rutin (138541.7µM TE/g) and synthetic antioxidant BHT was found to be 139722.2µM TE/g. The extract has the potential to scavenge the radical. ABTS produced by potassium persulfate was evaluated in comparison to a standard dose of trolox. In general, the presence of endogenous antioxidants such as phenolic compounds in medicinal plants may be valuable in protecting chemicals against oxidative damage[12]. In phenolics with large molecular weights, or tannins, have been found to have a greater capacity to squelch free radicals (ABTS)[24].

Superoxide Radical Scavenging Activity

The superoxide anion scavenging activity of leaves of *Piper wightii* established at a concentration of 100µg/mL is exhibited in Table No.2. The extracts are established to have the capacity to scavenge the superoxide radical found generated in the riboflavin-NBT-light system *in vitro*. The ability of an ethanol extract to scavenge superoxide radicals (44.84%) followed by water (31.22%) is prominent when compared to rutin (74.7%) and BHT (74.2%). The ethanol extract of leaves projected significant ($p > 0.05$) and extreme movement when compared to other solvent extracts. In biological systems, the hydroxyl radical is a very harmful free radical that is capable of causing significant damage to practically every single molecule located in living cells[25]. In the field of free radical pathology, it has been regarded as an extremely harmful group. DNA, lipids, and proteins may get oxidatively damaged by the highly reactive hydroxyl radicals[26].

Antibacterial activity of plants extract

Antibacterial activity of *Piper wightii* leaf different extracts of chloroform, ethyl acetate, ethanol and hot water extract were checked against five human bacterial pathogens. The results of the antibacterial activity of different extracts are presented in Table No.3 and Plate No.1. Generally, the ethanol extract of the leaf sample showed a significant zone of inhibition for *Klebsiella pneumonia* (22.03mm), *Escherichia coli* (22.35mm) and *Salmonella typhimurium* (25.23mm), *Bacillus subtilis* (22.3mm), *Staphylococcus aureus* (20.37mm) at a concentration of 20mg/mL. The widest zone of inhibition was observed in the ethanol extract of *Piper wightii* leaf extract, while the value dropped constantly for ethyl acetate, hot water and chloroform. The results revealed that both the fruit extracts showed promising antibacterial activity. Vibrio cholera is most effectively stopped by a *Piper wightii* leaf extract made of ethanol. Because microbial infections are a health problem all over the world and plants could be a source of antimicrobial agents[27], plants have been a good source of natural products for keeping people healthy and preventing infections. The World Health Organisation (WHO) says that medicinal plants are the best place to get a wide range of drugs and active compounds. So, these plants should be studied to find out more about their properties, safety, and effectiveness[28].

***In vitro* Anti-Inflammatory activity**

Membrane stabilization assay

The anti-inflammatory activities were expressed in percentage inhibition of leaf extracts of *Piper wightii* .were shown in Figure No.3. Among the various extracts leaf ethanol extracts of *Piper wightii* (63.69%) showed higher inhibition activity. Anti-inflammatory activity of the different extracts as follows as ethanol > ethyl acetate > hot water > chloroform > petroleum ether. During the inflammation, lysis of lysosomal membrane takes place where it releases enzyme components that produce various disorders[29]. NSAID either inhibits the release of lysosomal enzymes or stabilize the lysosomal membrane[30]. When RBC is exposed to injurious substances (hypotonic medium, heat, methyl salicylate and phenyl hydrasine) the lysis of the RBC membrane takes place with hemolysis and oxidation of haemoglobin[31]. Since human RBC membranes are similar to lysosomal membranes, the inhibition of hypotonicity and heat induced lysis are taken as measures to study the mechanism of anti-inflammatory activity[32].

GC-MS analysis

The chemical composition of *Piper wightii* leaf ethanol extracts was subjected to GC-MS analysis. The chemical compounds were analysed and

identified by comparing the retention times and their mass spectra with those in the NIST (National Institute of Standards and Technology) library. The identified compounds with their retention time (RT/ min.), molecular formula and molecular weight (m/z) are presented (Figure No.4 and Table 4). GC-MS analysis showed the presence of E, E, Z-1, 3, 12-Nonadecatriene-5, 14-diol in an ethanol extract of leaves with a peak area of 8.38%**.** The drugs anticancer, antidote, antitumor, and cytochrome-P450-2E1-inhibitor are also made from this substance. C-Teleopeptide excretion, endothelial leukocyte adhesion, deoxypyridinoline excretion, epinephrine production, and oxalate excretion are all decreased[33].

Table No.1: Phenolic, tannin and flavonoids content of *Piper wightii* leaf extracts

S.No	Extracts	Phenolic GAE/g extract	Tannin GAE/g extract	Flavonoids RE/ g extract
1	Petroleum ether	60.23 ± 1.82^{d}	59.16 ± 2.21^{d}	12.50 ± 4.71
2	Chloroform	59.35 ± 4.32	57.98 ± 5.54	28.76 ± 3.56^{d}
3	Ethyl acetate	96.78 ± 5.70^{c}	91.87 ± 6.76^{c}	174.88 ± 5.92^{a}
4	Ethanol	336.84 ± 5.47^{a}	331.52 ± 5.30^{a}	76.04 ± 0.65^{b}
5	Hot Water	120.76 ± 1.01^{b}	117.87 ± 2.43^{b}	55.63 ± 0.53^{c}

Values are mean of triplicate determination (n=3) ± standard deviation, statistically significant at $p < 0.05$ where $^{a}>^{b}>^{c}>^{d}$ in each column.

Table No.2: ABTS scavenging activity and Superoxide radical of *Piper wightii* Leaf Extracts

S.No	Samples	Extracts	ABTS scavenging Activity (µM TE/g extract)	Superoxide radical scavenging activity (%)
1	Leaf	Petroleum ether	24409.72 ± 1725.31	19.12 ± 1.48
		Chloroform	32812.5± 1900.86	23.2 ± 1.82
		Ethyl acetate	58298.61 ± 885.92^{d}	23.81 ± 1.34^{d}
		Ethanol	64166.67 ± 579.97^{c}	44.84 ± 0.58^{b}
		Hot Water	53333.33 ± 1201.3	31.22 ± 0.82^{c}
2	Standard	Rutin	138541.7 ± 416.66^{b}	74.7 ± 0.25^{a}
		BHT	139722.2 ± 636.46^{a}	74.2 ± 0.1^{a}

Values are mean of triplicate determination (n = 3) ± standard deviation, TE- Trolox Equivalents, statistically significant at $p < 0.05$ whereas $^{a}>^{b}>^{c}>^{d}$in each column.

Table No.3: Anti-bacterial activity of *Piper wightii* leaf extracts

S.No	Solvent	Concentration (mg/mL)	Diameter of Zone of inhibition (mm)				
			Gram Negative			Gram Positive	
			Klebsiella pneumonia	***Escherichia coli***	***Salmonella typhimurium***	***Bacillus subtilis***	***Staphylococcus aureus***
1	Negative Control		-	-	-	-	-
2	Chloroform	20	15.03 ± 0.03	18.33 ± 0.08^{d}	17.28 ± 0.17^{c}	18.28 ± 0.22^{d}	14.22 ± 0.13^{d}
3	Ethyl acetate	20	19.15 ± 0.14^{c}	21.42 ± 0.22^{c}	21.27 ± 0.13^{b}	21.3 ± 0.29^{b}	18.29 ± 0.22^{c}
4	Ethanol	20	22.03 ± 0.02^{b}	22.35 ± 0.06^{b}	25.23 ± 0.20^{a}	22.3 ± 0.32^{a}	20.37 ± 0.24^{b}
5	Hot Water	20	15.46 ± 0.14^{d}	18.24 ± 0.33^{d}	17.28 ± 0.20^{c}	20.37 ± 0.22^{c}	14.17 ± 0.12^{d}
6	Positive Control (Tigecycline)	15 mcg	22.36 ± 0.05^{a}	23.19 ± 0.20^{a}	21.36 ± 0.27^{b}	22.32 ± 0.15^{a}	22.24 ± 0.10^{a}

Values are mean of triplicate determination (n = 3) ± standard deviation, TE- Trolox Equivalents, statistically significant at $p < 0.05$ whereas $^{a}>^{b}>^{c}>^{d}$ in each column.

Table No.4: GC-MS profile of ethanol extract of *Piper wightii* leaf extract

S.No	Compound Name	Formula	RT	Peake Area %
1	1-Pentanol	$C_5H_{12}O$	3.7953	1.38
2	Peroxide, dimethyl	$C_2H_6O_2$	4.8836	1.10
3	4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl-	$C_6H_8O_4$	11.9303	1.02
4	3-Allyl-6-methoxyphenol	$C_{10}H_{12}O_2$	17.9562	1.16
5	Diethyl Phthalate	$C_{12}H_{14}O_4$	22.3269	1.0
6	6-Isopropenyl-4, 8a-dimethyl-1, 2, 3, 5, 6, 7, 8, 8 aoctahydro- naphthalen-2-ol	$C_{15}H_{24}O$	23.3676	1.12
7	CIS-1-Chloro-9-octadecene	$C_{18}H_{35}Cl$	24.8507	2.88
8	1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	$C_{16}H_{22}O_4$	25.9251	2.53
9	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	27.2114	6.15
10	1-Octadecanol	$C_{18}H_{38}O$	28.5127	3.95
11	E, E, Z-1, 3, 12-Nonadecatriene-5, 14-diol	$C_{19}H_{34}O_2$	29.2449	8.38
12	Bacteriochlorophyll-c-stearyl	$C_{52}H_{72}MgN_4O_4$	30.6841	1.32
13	Tetracosane	$C_{24}H_50$	31.7422	1.49
14	Pentacosane	$C_{25}H_{52}$	32.6439	1.40

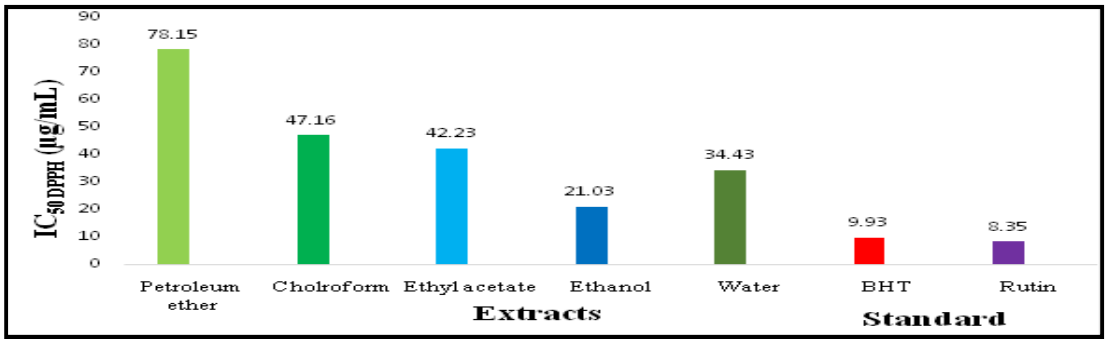

Figure No.1: DPPH radical scavenging activity of *Piper wightii* leaf extracts

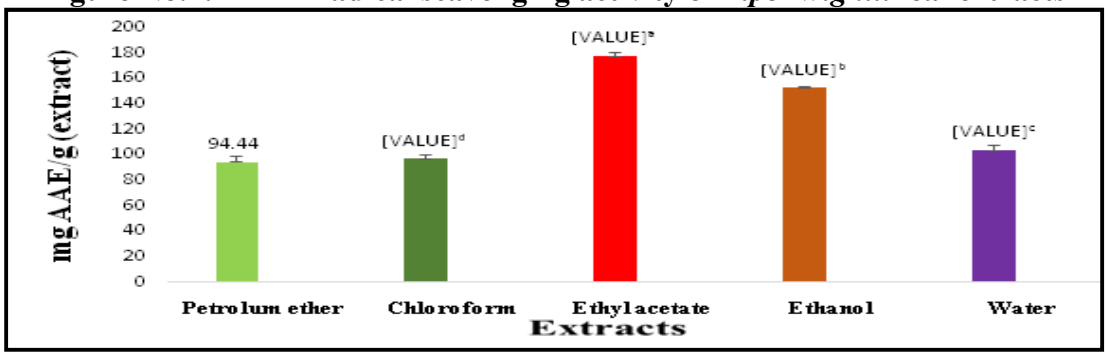

Figure No.2: Phosphomolybdenum Assay of *Piper wightii* Leaf Extracts

Values are mean of triplicate determination (n=3) ± standard deviation, statistically significant at $p < 0.05$ where $^{a}>^{b}>^{c}>^{d}$

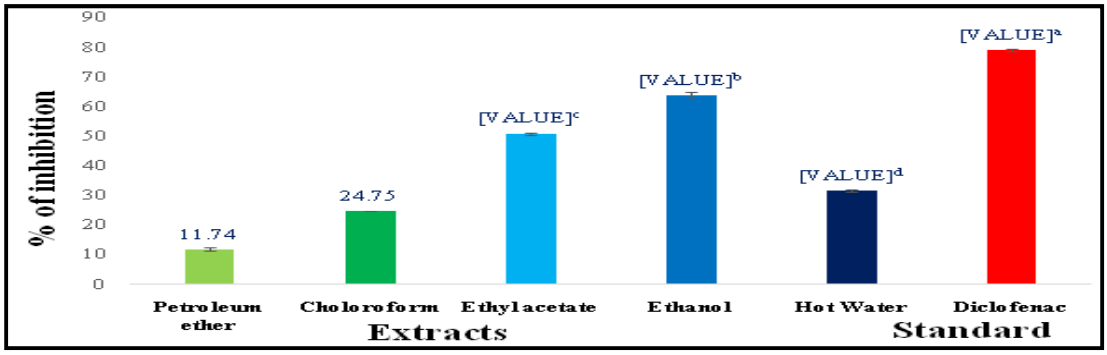

Figure No.3: *In vitro* Anti-Inflammatory activity of *Piper wightii* leaf extracts

Values are mean of triplicate determination (n = 3) ± standard deviation, TE- Trolox Equivalents, statistically significant at p <0.05 whereas $^{a}>^{b}>^{c}>^{d}$.

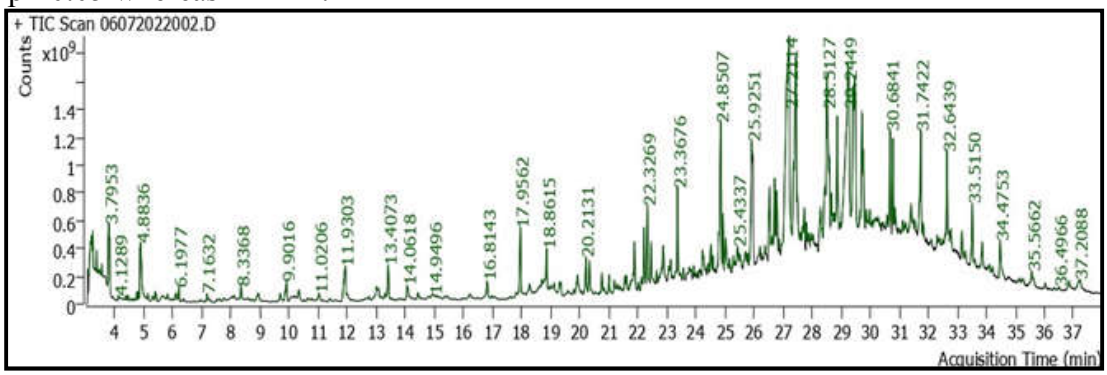

Figure No.4: GC-MS profile of ethanol extract of *Piper wightii* leaf extract

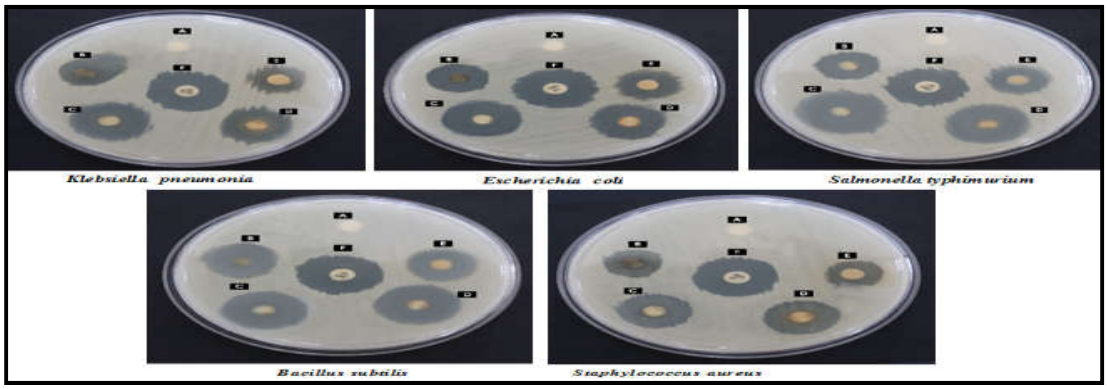

Plate No.1: Anti-bacterial activity of *Piper wightii* leaf Extracts

A: Negative Control (DMSO), B: Chloroform, C: Ethyl acetate, D: Ethanol, E: Hot water, F: Positive Control (Tigecycline)

CONCLUSION

From the results, it could be concluded that the ethanol extract of *Piper wightii* leaf exhibited strong antioxidant, anti-bacterial and anti-inflammatory properties. With the changing global scenario towards the use of non-toxic herbal products, there is an urgent need for the development of much safer drugs from *Piper wightii* for inflammation related diseases. In the future, researchers will have a promising scope in the isolation, identification and characterization of active chemical components, which will yield a better understanding of the bioactive products and subsequently help in the management of disease.

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CONFLICT OF INTEREST

We declare that we have no conflict of Interest.

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