

Evaluation of Preliminary Phytochemical Screening, Antioxidant activity, Isolation and characterization of bioactive compounds in *Waltheria indica* Linn.

Abstract

Objective: *Waltheria indica* Linn, a rare endemic medicinal plant in South India, was studied for phytochemical screening, antioxidant activity, and bioactive compound isolation from a hydroalcoholic extract.

Methods: *In-vitro* methods such as 1, 1-diphenyl-2-picrylhydrazole (DPPH), 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and Reducing Power Assay were used in the study. The method of compound isolation was used to isolate pure compounds. Phytochemical components were screened for particular extracts such as hydroalcoholic, ethyl acetate and chloroform extracts at different concentrations (50-1000 µg / mL).

Results: *Waltheria indica* plant phytochemical analysis in hydro-alcoholic extracts, Linn finds alkaloids, flavonoids, hormones, terpenoids, phenols, tannins, and carbohydrates, among other biochemicals. Ethyl acetate included flavonoids, hormones, terpenoids, phenols, and carbohydrates, but no phlobatannine. In Chloroform extract, there was a very small amount of flavonoid, carbohydrate, and resin phytoconstituents. 1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity (437.73 g / ml), ABTS assay (381.72 g / ml), and power reduction assay (550.14 g / ml) antioxidant activity Two bioactive compounds were isolated they are (E, 7R, 11R)- 3,7,11,15 - tetramethyl hexadec-2- en-1-ol Phytol and 2-Methyl hexadecan- 1- ol 1- Hexadecanol (2-methyl).

Conclusion: The current study confirms the presence of various phytochemicals in *Waltheria indica* hydro-alcoholic extract, as well as its strong antioxidant ability. It can be studied further in the field of pharmaceutical science with the aid of active compound.

Keyword: Column chromatography, TLC, Phytoconstituents, DPPH radical scavenging activity, ABTS assay, Reducing power assay and *Waltheria indica* Linn.

1. Introduction

Plants have long been recognized as a valuable source of novel therapeutic compounds for the treatment of a variety of diseases, and their use in conventional medicine has been reported. Medicinal plants and their properties are used to treat a wide range of

diseases all over the world. The use of medicinal plants in traditional medicines is on the rise nowadays, particularly in the field of health. According to the World Health Organization (WHO), the majority of the world's population depends on traditional medicine for health care, so medicinal plant discovery is on the rise to meet the group's needs. The high effectiveness of medicinal plants and their active ingredient as a therapeutic medication with no negative side effects has sparked a surge in interest in them. It has been found to be an antioxidant (Manurung *et al.*, 2017; Neagu *et al.*, 2018; Bogucka-Kocka *et al.*, 2018), antibacterial (Mostafa *et al.*, 2018), antihypertensive (Sultana, 2017), and anticancer agent in medicinal plants all over the world (Oyenihi, 2019). Comprehensive research on different plant species and their therapeutic principles are now revealing modern medicine all over the world. Phytochemicals found in plants have a wide range of bioactivity, including antioxidant, anti-inflammatory, and anti-cancer properties. Approximately 25% of the active ingredient has been identified from plants used as prescription medicines at this time (Gill *et al.*, 2011).

Antioxidants are compounds that act as scavengers or blockers of reactive oxygen species (Nimse and Pal, 2015). Natural antioxidants improve plasma antioxidant capacity and reduce disease danger (Shekhar *et al.*, 2011). Different portions, such as seeds, leaves, stems, and root bark, are known to contain significant amounts of phytoconstituent substances, such as phenols, flavonoids, and tannins, which can inhibit excessively produced free radicals and thus function as antioxidants (Samatha *et al.*, 2012). Because of the growing awareness of herbal remedies as potential sources of phenolic oxidants, continued research among plant secondary metabolites for natural antioxidants has become increasingly important in recent years (Aliyu *et al.*, 2010). Many life-threatening diseases are linked to lipid, protein, and DNA oxidation, including cancer (Galano *et al.*, 2018), atherosclerosis (Yang *et al.*, 2017), heart disease (Abushouk *et al.*, 2017), diabetes (Cacciapuoti, 2016), preeclampsia (Hansson *et al.*, 2015), and neurodegenerative disorders such as Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, celiac disease (Niedzielska *et al.*, 2016; Manoharan *et al.*, 2016; Manoharan *et al.*, 2016; Uddin *et al.*, 2016; Uddin *et al.*, 2017) and Parkinson's disease (Abushouk *et al.*, 2017).

The extraction of medically essential compounds from plants used in traditional medicinal practices has become possible thanks to recent developments in science. Phytochemicals, which are bioactive compounds found in plants, are present. These phytochemicals are extracted from a variety of plant parts, including leaves, barks, seeds, seed coats, flowers, roots, and pulps, and are thus used as direct medicinal agents (Ingle *et al.*,

2017). Extracts have been shown to be biologically active in both in vitro and in vivo test systems in some cases (Van Wyk and Wink, 2004). Certain plant-derived compounds work well in conjunction with others, while others work well on their own (Balunas and Kinghorn, 2005). Separating substances, on the other hand, remains a difficult and time-consuming process. The isolation of bioactive compounds is usually accompanied by the determination of the presence of specific compounds within plant extracts using a variety of bioassays. *Waltheria indica* Linn is the name of the plant. The Sterculiaceae family includes the sleepy morning, also known as velvet grass, marshmallow, monkey bush, boater bush, leather coat, buff coat, and many other names (Burkill, 2000). It is a perennial shrub with stalked leaves with narrow and irregularly toothed margins that grows up to 500 mm long. Yellow flowers grow in clusters. Its distribution and habitat are commonly found in subtropical and tropical areas, in scrub forests, flooded savannas, river banks, sandy or clay soils, and in disturbed or degraded soils (Saunders, 2007). (Saunders, 2007). The aim of this study was to look at the phytochemical components and antioxidant activity of *W.indica* in three different extracts.

2. Materials and Methods

2.1 Collection of Plant Materials

Fresh *Waltheria indica* samples were randomly collected from the Yercaurd Hills in Tamil Nadu. The samples were washed under running tap water, air dried, and homogenized into fine powder before being placed in airtight bottles in the refrigerator.

2.2 Preparation of Extracts

The Soxhlet extraction method was used to prepare the Crude Sample Extract. Approximately 20 gm of powdered *Waltheria indica* sample content was uniformly wrapped in a thimble and collected separately with 250 ml of hydro-alcohol, ethyl acetate, and chloroform. The extraction process must be continued for a total of 24 hours or until the solvent in the extractor's syphon tube has turned colorless. The extract was then transferred to a beaker and put on a hot plate, where it was heated at 30-40°C until all of the solvent had evaporated. Dry extract was kept in the refrigerator at 4°C for future use.

2.3 Phytochemical Screening

Waltheria indica hydro-alcohol, ethyl acetate, and chloroform extracts were subjected to preliminary phytochemical analysis using the standard methods defined by Brain and Turner in 1975 and Evans in 1996.

2.4 Antioxidant activity

2.4.1 DPPH Radical Scavenging Activity

The Molyneux method was used to perform the DPPH radical scavenging (2004). Equivalent volume of test sample in different concentrations of methanol was applied to 1,0 ml of 100,0 M DPPH solution in methanol and incubated in the dark for 30 minutes. A spectrophotometer set to 514 nm showed an increase in coloration in terms of absorbance. Instead of the test sample, 1.0 ml of methanol was added to the control tube. Ascorbic acid in various amounts was used as a reference drug.

2.4.2 ABTS radical scavenging activity

Re *et al.*, 1999 estimated the extract's radical-scavenging process. 5 ml of 14 mM ABTS solution and 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution were mixed for 16 hours in the dark at room temperature. The plant extract was homogenized in 1 ml of ABTS solution at various concentrations, and its absorption at 734 nm was measured. In each assay, ethanol blanks were used, and all measurements were taken for at least 6 minutes. Similarly, 950 μ l of ABTS+ solution and 50 μ l of BHT were combined to make the normal group reaction mixture. The ability to scavenge ABTS was expressed as IC_{50} (μ g / ml) in terms of anti-radical behavior.

2.4.3 Reducing power assay

With 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1 percent potassium ferricyanide, the sample was spiked with Ascorbic acid solutions. For 20 minutes, the mixture was kept in a 50°C water tank. The resulting solution was quickly cooled before being spiked with 2.5 mL of 10% trichloroacetic acid and centrifuged for 10 minutes at 3000 rpm. The supernatant (5 mL) was combined with 5 mL distilled water and 1 mL ferric chloride (0.1%) and incubated for 10 minutes. At 700 nm, the spectrophotometer observed absorption. The extract's absorbance concentration was calculated by plotting the absorbance graph at 700 nm against the extract's concentration. Ascorbic acid was used as a benchmark. The higher the absorbance, the greater the reducing force (Oyaizu, 1986).

2.4.4 Formula for Percentage of inhibition

Percentage of inhibition was calculated from the equation

$$[(\text{Absorbance of control} - \text{Absorbance of test}) / \text{Absorbance of control}] \times 100.$$

IC_{50} value was calculated using Graph pad prism 5.0.

2.5 Chromatographic techniques

2.5.1 Thin-layer chromatography (TLC) and Column chromatography (CC)

TLC is a short, simple, and inexpensive procedure that tells the researcher how many components are present in a blend. TLC is sometimes used to aid in the detection of a compound in a mixture when its R_f is compared to that of a known compound (Shahverdi *et al.*, 2007). The extract residue (10 g) was added to a silica gel column (200 g, 100cm x 3.5 cm) to separate the active compound from the crude extracts using hexane as a solvent, which increased the polarity, followed by chloroform, and fractions (100 mL) were collected. Using hexane, acetone and methanol (97:2:1) as mobile phases, the obtained fractions were concentrated and tracked using TLC. Merck's TLC plates (TLC Silica gel 60 F254) were used to observe the separation of individual compounds as a single spot was trimmed and the origin was marked with a straight line. $R_f = \text{Distance travelled by material} / \text{Distance travelled by solvent front}$ is the formula used to calculate R_f .

2.5.2 Structure Determination

The structure of natural products is determined using data from a variety of spectroscopic techniques, including infrared (IR), nuclear magnetic resonance (NMR), and mass spectroscopy. The fundamental idea behind spectroscopy is that electromagnetic radiation is transferred into an organic compound that absorbs some but not all of it. The sum of absorption of electromagnetic radiation can be used to create a continuum. Such bonds have a spectrum that is unique to them. These spectrums can be used to determine the natural compound's composition. Scientists typically use spectra from three or four regions for structural clarification: infrared (IR), radio frequency (FTIR), and mass spectra (Popova *et al.*, 2008).

3. Results and Discussion

3.1 Phytochemical analysis

As shown in Table 1, phytochemical screening of various raw leaf extracts of *Waltherica indica* revealed the existence of some secondary metabolites. Alkaloids, flavonoids, steroids, terpenoids, phenols, tannins, and carbohydrates have been found in hydro-alcoholic extracts, while flavonoids, steroids, terpenoids, phenols, tannins, and carbohydrates have been found in ethyl acetate. In Chloroform extract, there was a very small amount of flavonoid, carbohydrate, and resin phytoconstituents. In comparison to the other two extracts, the hydro-alcoholic extract has higher activity (Figure: 1 – 3). For more study, hydro-alcoholic extracts are used. Phytochemicals are secondary metabolites that help plants deal with short-term or long-term threats to their climate, and they're important for human

health (Molyneux *et al.*, 2007). Anti-cancer drugs derived from plants, such as asetoposide and taxol, have been used in clinical trials for years and are important in the creation of new human drug entities (Phillipson, 2001).

Table 1: Qualitative phytochemical analysis of *Waltherica indica* extract

Phytochemicals	Observations	Extracts		
		Hydro alcohol	Ethyl acetate	Chloroform
Alkaloids Mayer's test	Cream color Reddish brown solution/ precipitate	+	-	-
Flavonoids H ₂ SO ₄ test	Yellow orange Reddish brown / Orange color precipitate	++	+	+
Steroids Liebermann- Burchard test	Violet to blue or Green color formation	++	+	-
Terpenoids Salkowski test	Reddish brown precipitate	++	+	-
Arthroquinone Borntrager's test	Pink color	-	-	-
Phenols Ferric chloride test	Deep blue to Black color formation White precipitate	++	+	-
Saponin	Stable persistent	-	-	-
Tannin	Brownish green / Blue black	+	-	-
Carbohydrates	Yellow / brownish / blue / green color	++	+	+
Oils & Resins	Filter paper method	-	-	+

Phytochemicals such as alkaloids, tannins, saponins, steroids, terpenoids, flavonoids, phlobatannine, and cardiac glycosides have been screened extensively in medicinal plants that lead to ethnomedicine (Edeoga *et al.*, 2005). They've been linked to the prevention and/or treatment of chronic diseases like heart disease, cancer, diabetes, and hypertension, as

well as other illnesses (Craig, 1997). Tannins, saponins, and cardiac glycosides are some of the most common secondary metabolites found in *Waltherica indica* aqueous and powdered root extracts (Zailani *et al.*, 2010). Phenolic compounds have redox properties and act as antioxidants, according to Soobrattee *et al.*, 2005. Antioxidants, antibacterial, anti-inflammatory, anti-allergic, and anti-mutagenic properties are all features of flavonoids (Iqbal and Kamariah, 2015). Cytotoxicity (Thite *et al.*, 2013), antimalarial (Dua, Gaurav *et al.*, 2013), and anti-inflammatory properties have all been identified for alkaloid (Augusto *et al.*, 2011). Steroids and triterpenoid compounds may be used as analgesics, as well as having antibacterial and insecticidal properties (Alexei *et al.*, 2009).

Figure 1: Qualitative phytochemical analysis of *Waltherica indica* Hydro-alcoholic Extract

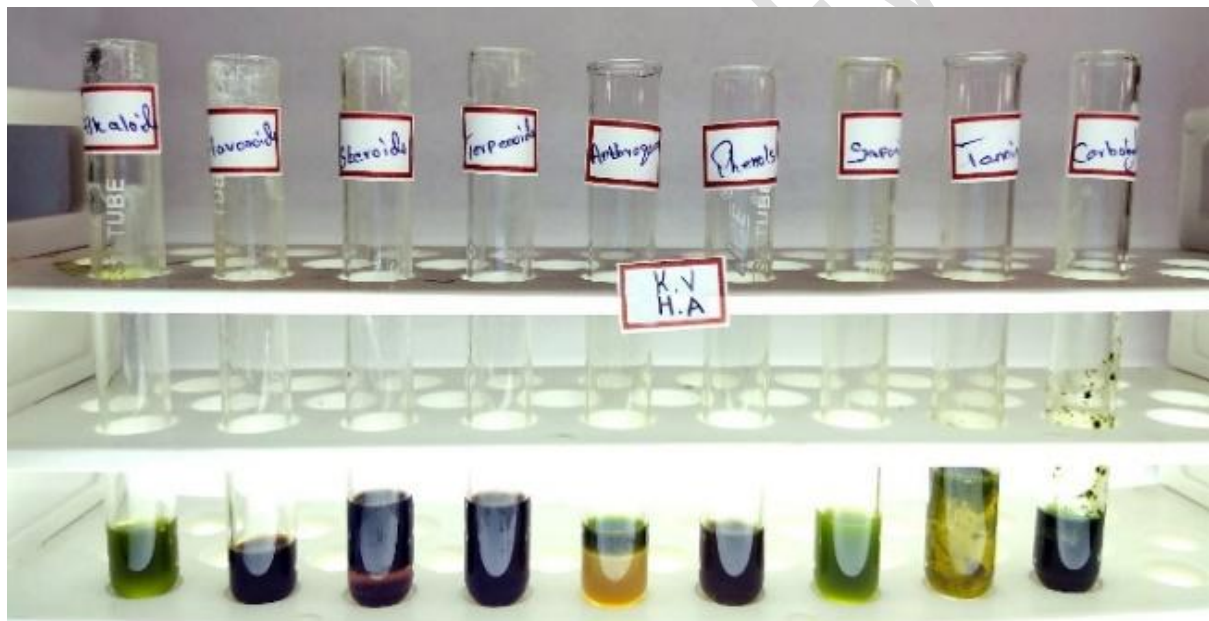


Figure 2: Qualitative phytochemical analysis of *Waltherica indica* Chloroform Extract



Figure 3: Qualitative phytochemical analysis of *Waltherica indica* Ethyl Acetate Extract

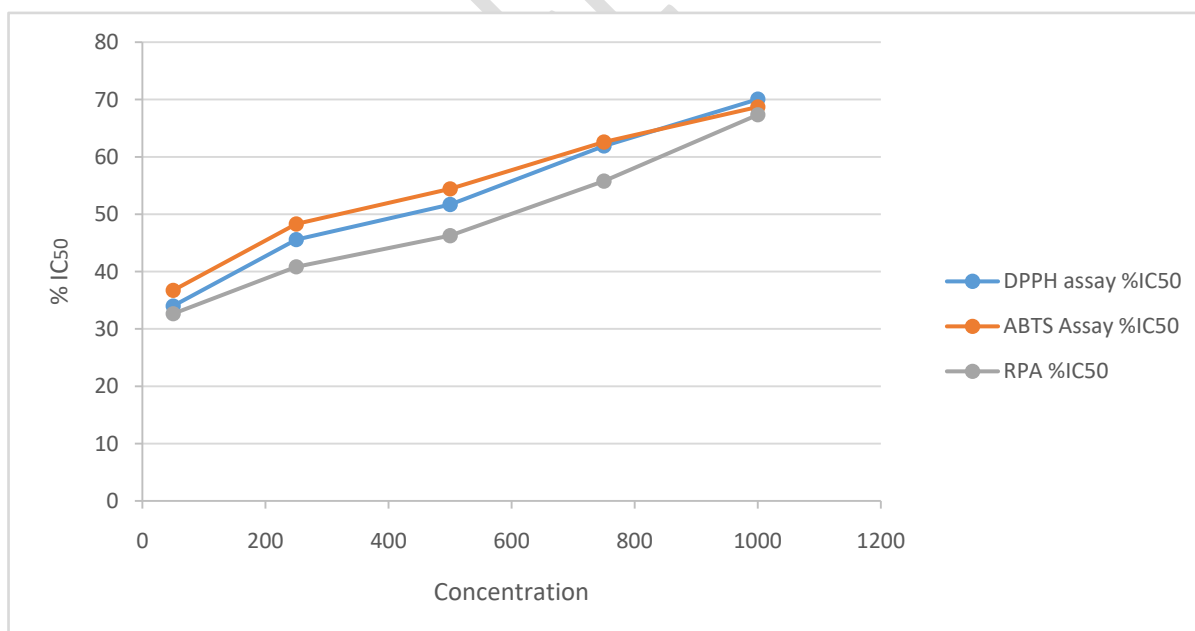


3.2 Antioxidant Activity

Antioxidant activity is checked for DPPH assay, ABTS assay and Power Reduction assay. Table and Figure show the results of the hydro-alcoholic extract test using the DPPH (2, 2-diphenyl-1-picrylhydrazide) free radical inhibition process. According to the table, the higher the sample concentration, the easier it was to cause a higher inhibition percentage (percent).

This was due to the sample's higher concentration, which resulted in a higher antioxidant content, which weakened free radical inhibition. We recently used IC_{50} (inhibition concentration) to demonstrate antioxidant activity, with IC_{50} specified as the value of an extract concentration that inhibits radical activity by about 50%. The percentage of radical interception was calculated using a linear regression equation that showed a connection between the concentration of the extract tested and the percentage of radical interception. The IC_{50} value showed a lower number than that associated with higher extract antioxidant activity (Molyneaux, 2004). 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity ($437,73 \mu\text{g} / \text{ml}$), ABTS assay ($381,72 \mu\text{g} / \text{ml}$), and power reduction assay ($550,14 \mu\text{g} / \text{ml}$) antioxidant activity (Figure: 4). The hydro-alcoholic extract of *Waltherica indica* has the strongest free radical scavenging activities and reduces the capacity of all plant extracts analyzed, indicating that they may be useful for the treatment of free radical diseases. The extracts were found to scavenge the $ABTS\bullet+$ radical more effectively than the DPPH radical scavenging and power reduction assay. It is well known that the antioxidant efficacy of a plant extract depends largely on both its composition and the test method (Wang *et al.*, 2013).

Figure 4: Antioxidant Activity of *Waltherica indica* Hydro-alcoholic extract



3.3 Column Chromatography

Chromatographic fractionation of the crude sample of *Waltherica indica* carried out in hydro-alcoholic extract. The hydro-alcoholic extract yielded a total of 35 compounds, with compound 10 and compound 16 showing the most activity. When compared to the other

compounds, these two have higher antioxidant activity (94.32 percent and 95.24 percent). The compound eluted from the hydro-alcoholic extract proved to be the most efficient solvent in terms of scavenging efficiency, with the lowest IC₅₀ values. As a result, the bioactive compounds responsible for antioxidant activity were extracted and purified from this extract.

The spectral analysis was carried out for compound 16 ((E, 7R, 11R) - 3, 7, 11, 15-tetramethyl hexadec-2- en-1-ol)/ Phytol (Figure: 5) and compound 10 (2- Methyl hexadecan-1- ol). In ((E, 7R, 11R)- 3,7,11,15- tetramethyl hexadec-2- en-1-ol (Figure: 6) the Molecular Formula is C₂₀H₄₀O, Molecular Weight is 296.531 g/mol, Molar Refractivity: 98.94 cm³. Whereas 2- Methyl hexadecan- 1- ol has its Molecular Formula C₁₇H₃₆O and Molecular Weight is 256.4671 g/mol.

Figure 5: Structure of Phytol

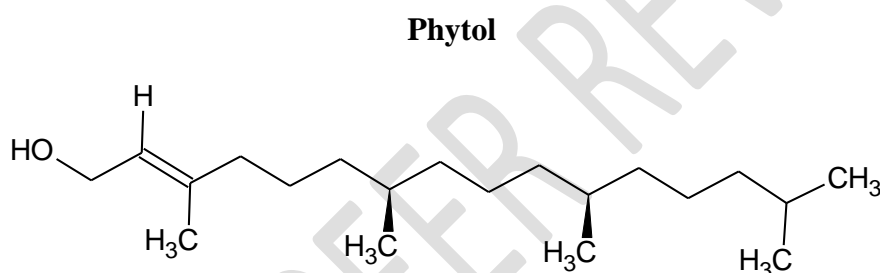
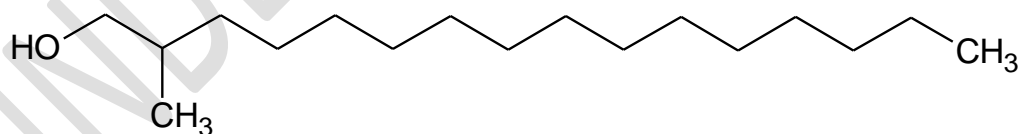


Figure 6: Structure of 2- Methyl hexadecan- 1- ol

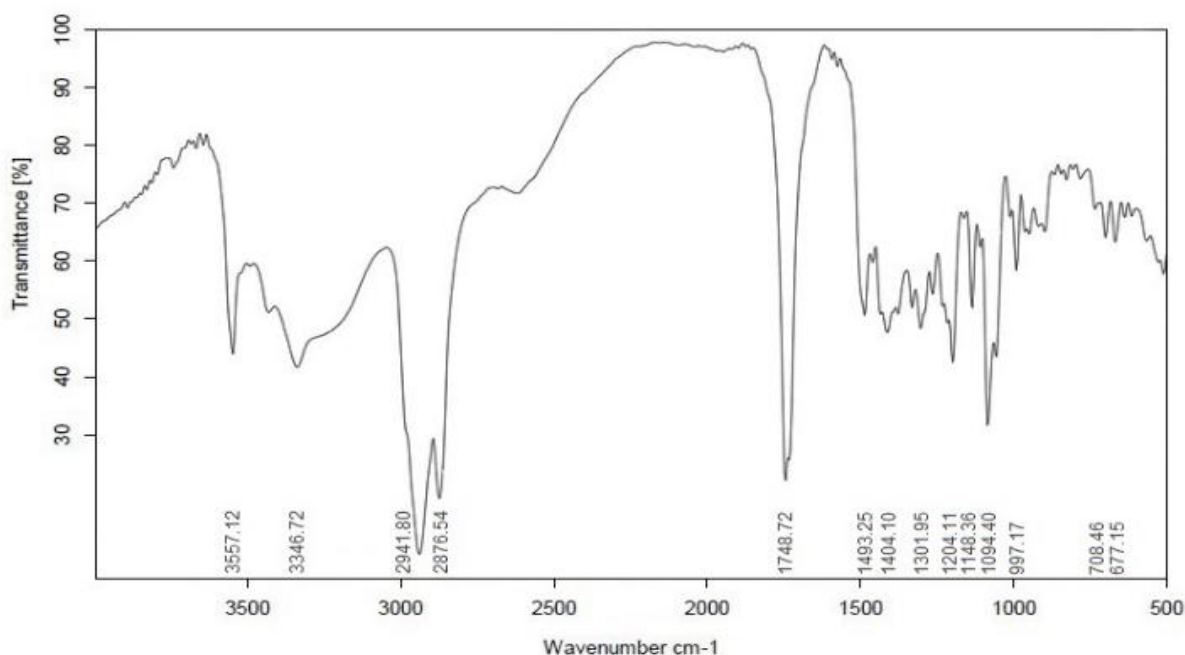


3.4 FTIR Analysis

The compound Phytol exhibited a characteristic band at 3557.12 cm⁻¹ indicating the presence of Inter molecular hydrogen bonded OH (Strong), 3346.72 cm⁻¹ indicating the presence of Primary amines (Weak to medium), 2941.80 cm⁻¹ indicating the presence of Vinyl terminal (medium), 2876.54 cm⁻¹ indicating the presence of Alkanes (medium), 1748.72 cm⁻¹ indicating the presence of Aromatic Methane (weak), 1493.25 cm⁻¹ indicating the presence of Alkanes (medium), 1404.10 cm⁻¹ indicating the presence of Quaternary compounds (Strong), 1301.95 cm⁻¹ indicating the presence of Primary amines (Strong),

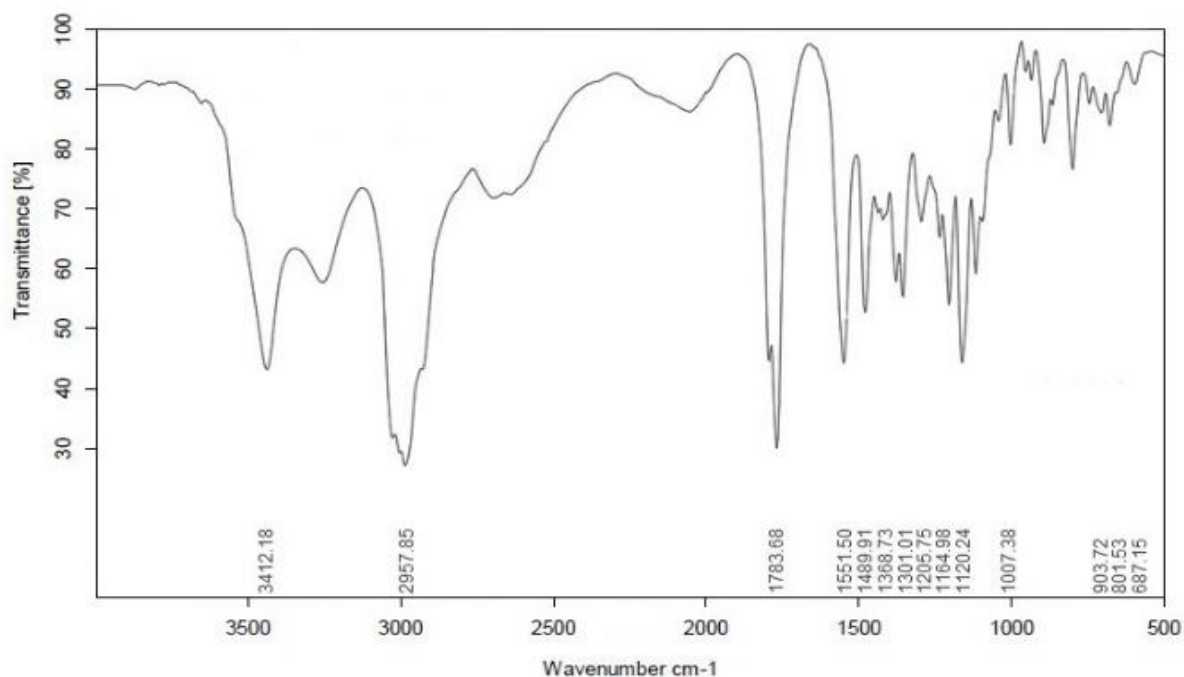
1204.11 cm^{-1} indicating the presence of Primary amines (Weak to medium), 1148.36 cm^{-1} indicating the presence of Aromatic esters (Very strong), 1094.40 cm^{-1} indicating the presence of Aliphatic esters (Very strong), 997.17 cm^{-1} indicating the presence of Cycloalkanes (medium), 708.46 cm^{-1} indicating the presence of Primary amines (weak) and 677.15 cm^{-1} indicating the presence of Meta disubstituted (Medium to strong) (Figure: 7).

Figure 7: FTIR analysis of Phytol and 2- Methyl hexadecan- 1- ol



In 2- Methyl hexadecan- 1- ol exhibited a characteristic band at 3412.18 cm^{-1} indicating the presence of Inter molecular hydrogen bonded OH (Strong), 2957.85 cm^{-1} indicating the presence of Alkanes (Strong), 1783.68 cm^{-1} indicating the presence of Acid peroxides (Very strong), 1551.50 cm^{-1} indicating the presence of Secondary amines (weak), 1489.91 cm^{-1} indicating the presence of Acids (medium), 1368.73 cm^{-1} indicating the presence of Aliphatic aldehydes (Very strong), 1301.01 cm^{-1} indicating the presence of Aromatic esters (very strong), 1205.75 cm^{-1} indicating the presence of Aromatic ketones (Strong), 1164.98 cm^{-1} indicating the presence of Isopropyl (Strong), 1120.24 cm^{-1} indicating the presence of Aliphatic esters (Very strong), 1007.38 cm^{-1} indicating the presence of Aromatic methane (weak), 903.72 cm^{-1} indicating the presence of Primary amines (medium), 801.53 cm^{-1} indicating the presence of Meta disubstituted (Very strong) and 687.15 cm^{-1} indicating the presence of Meta disubstituted (Medium to strong) (Figure: 8).

Figure 8: FTIR analysis of 2- Methyl hexadecan- 1- ol



3.5 Mass Analysis

EI-MS m/z: The molecular ion peak is (M+) 296. The other fragments are 57, 71, 81, 95, 111, 123, 137, 196, 221, 249, and 278 in Phytol where as in 2- Methyl hexadecan- 1- ol
EI – MS m/z: The molecular ion peak is (M+) 256. The other fragments are 60, 73, 83, 97, 129, 157 and 213. (Figure: 9,10)

Figure 9: Mass analysis of Phytol and 2- Methyl hexadecan- 1- ol

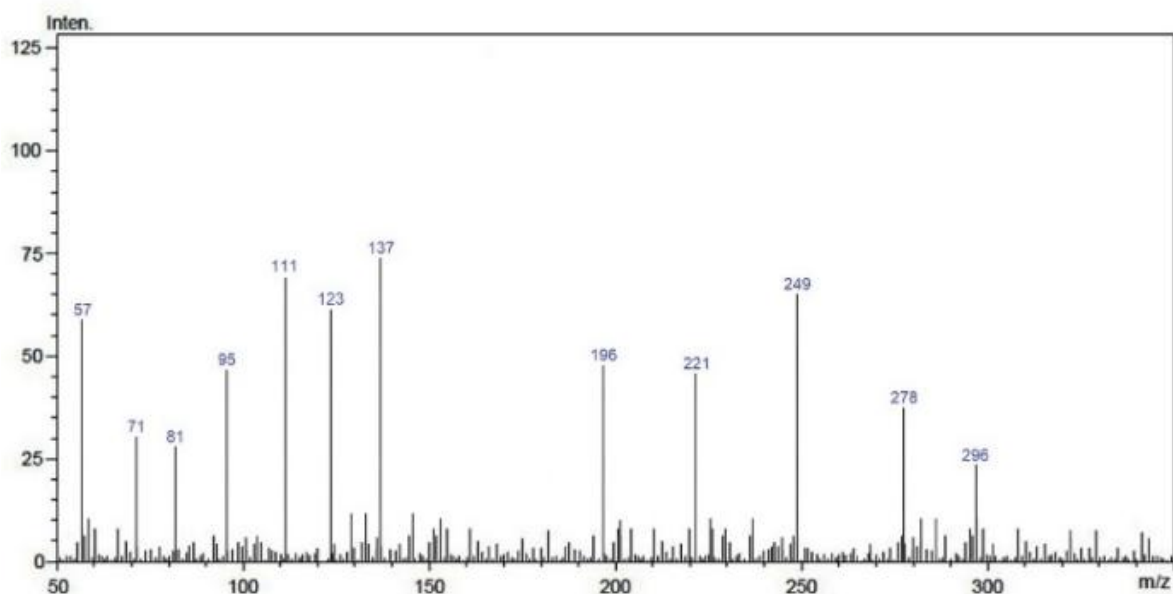


Figure 11: H^1 NMR Analysis of Phytol and 2-Methyl hexadecan-1-ol

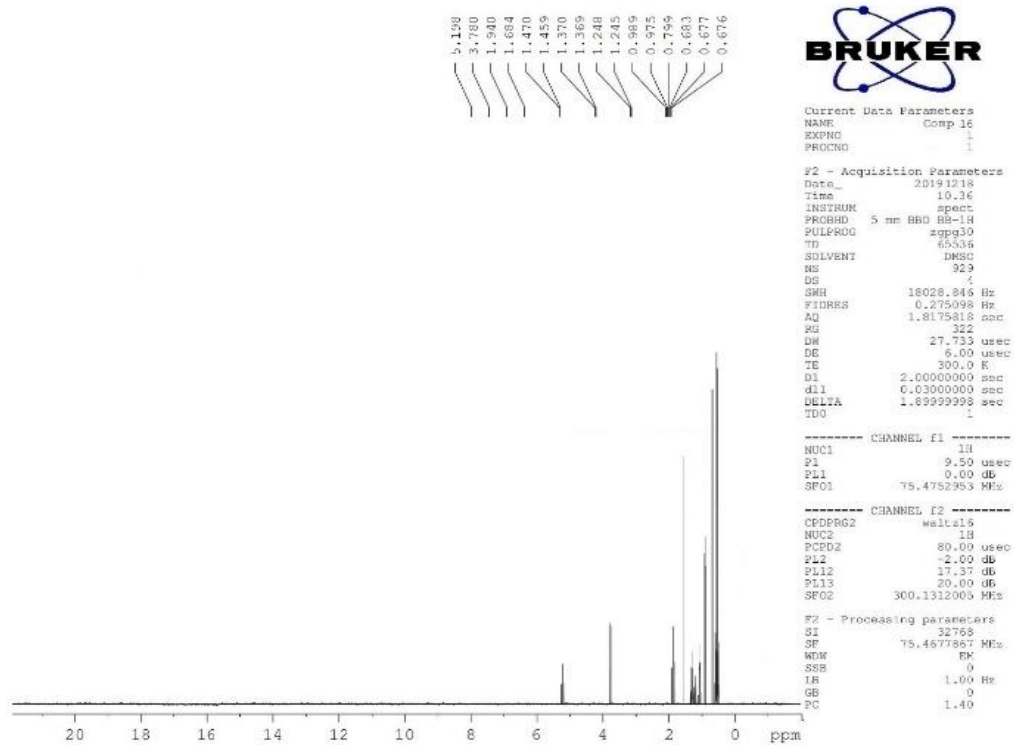
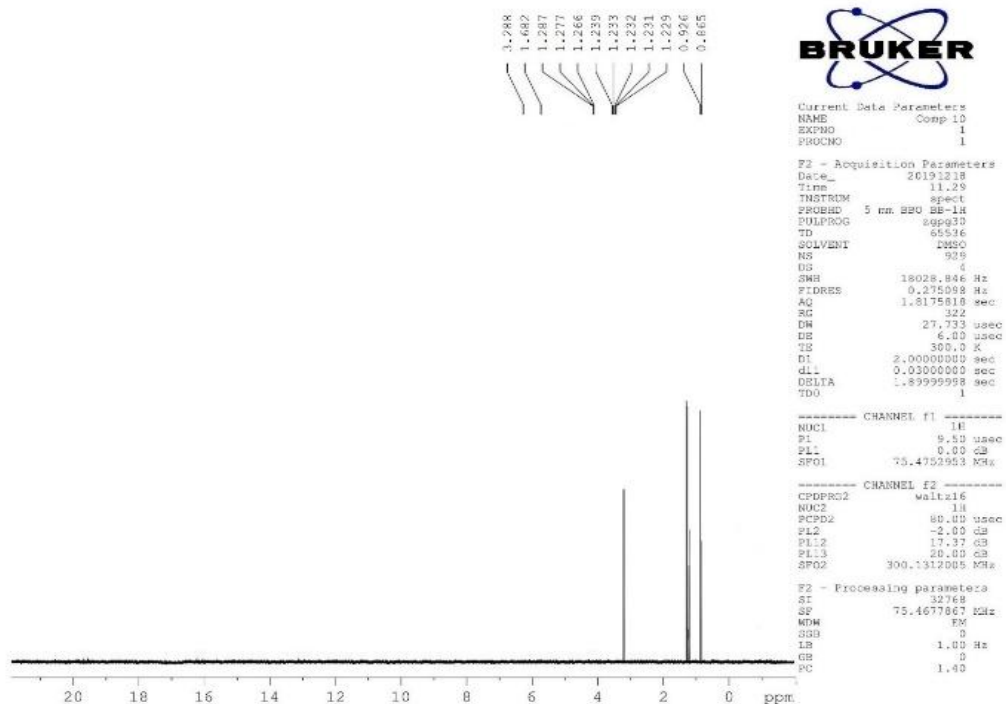


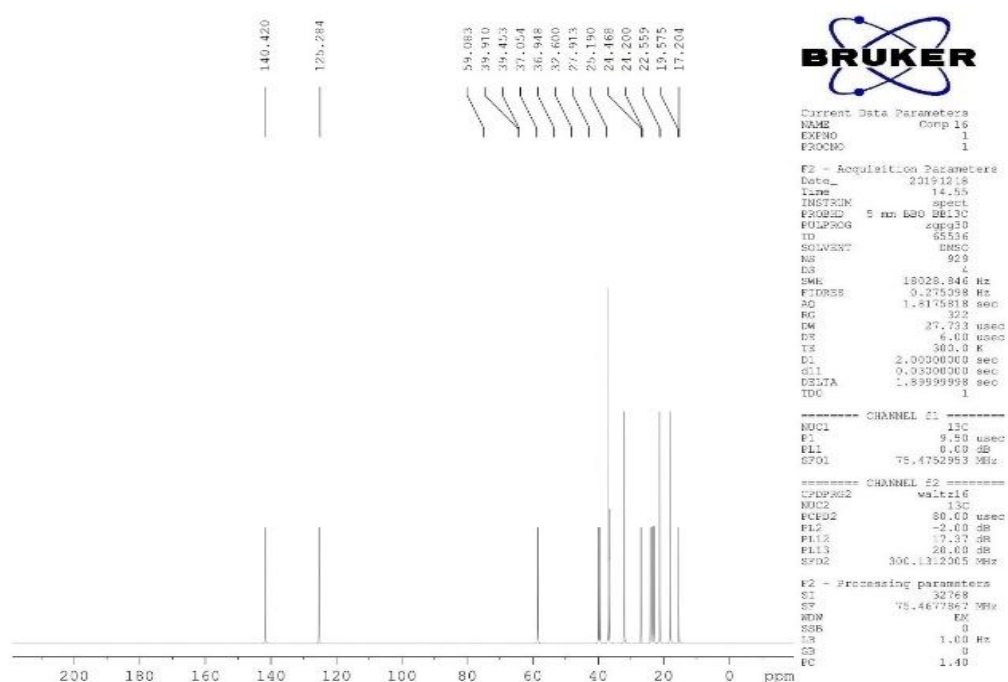
Figure 12: H^1 NMR Analysis of 2- Methyl hexadecan- 1- ol



3.7 C^{13} NMR Analysis

The compound Phytol exhibited 17.204, 19.575, 22.558, 24.200, 24.468 as CH₃CO, 25.190, 27.913 as R₃CH, 32.600, 36.948, 37.054, 39.452, 39.910 as RCH₂NH₂, 59.083 as RCH₂O, 125.284 as C in aromatic rings and 140.420 as C=C (in alkanes) (Figure: 13). where as in 2- Methyl hexadecan- 1- ol 14.001, 16.477 as CH₃CO, 22.619, 26.786, 28.323, 28.958, 29.054, 29.359 as R₃CH, 32.403, 33.252 as RCH₂NH₂ and 68.094 as RCH₂O (Figure: 14).

Figure 13: C¹³ NMR Analysis of Phytol and 2- Methyl hexadecan- 1- ol



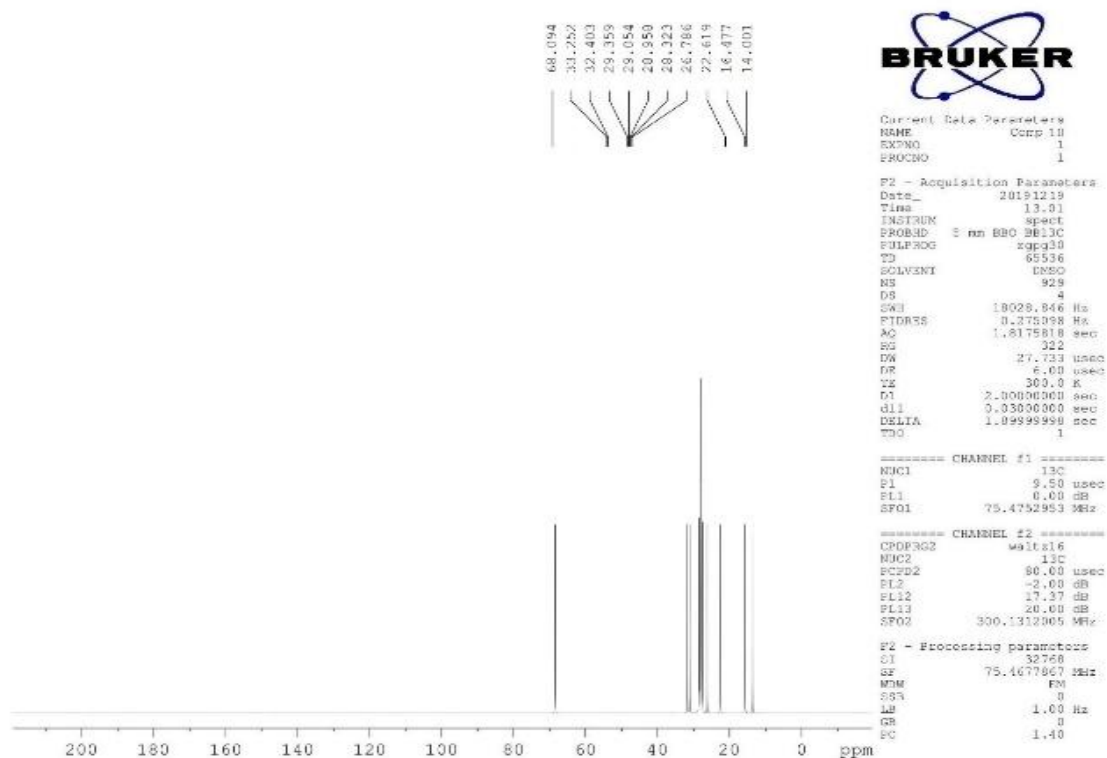


Figure 14: C^{13} NMR Analysis of 2- Methyl hexadecan- 1- ol

4. Conclusion

The isolation of natural products derived from plant extracts presents a major challenge for the identification and characterization process because they usually contain various mixtures of components with different polarities. Extraction is crucial in the isolation and characterization of different natural products. To remove natural products, they must be filtered using a mixture of chromatographic and non-chromatographic techniques, as well as a variety of other purification methods. The aim of this study was to see if *Waltherica indica* leaves, which are commonly used in traditional medicine, could be good sources of natural antioxidants. Hydro-alcoholic extracts contain higher amounts of phyto-constituents than ethyl acetate and chloroform extracts, according to phytochemical tests for various constituents. For hydro-alcoholic extract, the DPPH radical scavenging assay, the ABTS assay, and the reduced power assay were used, with the ABTS assay showing better operation. Phytol and 2- Methyl hexadecan-1- ol are the isolated compounds that can be used to make drugs.

NOTE:

The study highlights the efficacy of "traditional medicin" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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