Full Length Research Article

© 2024, Centre for Advanced Research in Agricultural Sciences
Research Journal of Agricultural Sciences
Volume 15; Issue 03 (May–Jun 2024); pp 789–795

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

Secondary Metabolites, *in vitro* Antioxidant and Anti-inflammatory Activity of *Crotalaria ramosissima* Roxb. Leaves

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Received: 01 Mar 2024; Revised accepted: 20 May 2024

Abstract

Crotalaria ramosissima leaves are among the important wild medicinal plant used by the tribes of the Western Ghats. However, this underutilized leaf remained unnoticed for its medicinal properties. Hence, the present study was undertaken to evaluate primary phytochemical, secondary metabolites by phenolic, tannin and flavonoid, antioxidant activity by DPPH, ABTS, FRAP assays, superoxide scavenging activity, Phosphomolybdenum, and anti-inflammatory activity by membrane stabilization method. leaf ethyl acetate extracts showed the maximum amount of phenolics (80.19 mg GAE/g extract), tannin (70.58 mg GAE/g extract) and flavonoids (111.55 mg RE/g). It also revealed the presence of ethyl acetate highest antioxidant properties by estimating DPPH (IC_{50} value of 34.46 µg/mL), ABTS (74263.9 µM TE/g), FRAP ethanol (32.29 mM Fe (II)/mg extract), Superoxide radical scavenging activity (54.79%,) and Phosphomolybdenum (145 mg AAE/g extract). The ethyl acetate extract (76.75%) showed a high degree of inhibition in the anti-inflammatory assay. *V. symplocifolium* leaf extracts have a tremendous amount of antioxidant potential, making them a good source of antioxidant supplements for food to protect against oxidative stress-related diseases, including inflammation.

***Key words*:** *Crotalaria ramosissima*, Antioxidant, Anti-inflammatory, *V. symplocifolium*, Phosphomolybdenum

Inflammation is often described as a multi-faceted physiological reaction of blood vessel tissues to potentially damaging stimuli. In addition, inflammation A wide variety of useful medications and nutritional supplements may be derived from plants and plant components. In terms of healthcare management, about three quarters of the world's population relies on plants and their products. Essential dietary components including carbs, protein, and fat are present in medicinal plants [1]. Polyphenolic substances have antioxidant properties because they can reduce free radicals, donate hydrogen, quench single oxygen molecules, and chelate metal ions. Because of these characteristics, they are able to prevent metals from generating free radicals [2-3]. Antioxidant chemicals work by preventing free radicals from oxidizing other molecules or starting or spreading such chain reactions. This protects the body and reduces oxidative damage, which is a major risk factor for many chronic illnesses, including diabetes, cancer, cancer, and heart disease [4].

Superoxide anion, hydrogen peroxide, hydroxyl radicals, and other reactive oxygen species (ROS) are scavenged by these antioxidants. Inducing oxidative stress, reactive oxygen species (ROS) target polyunsaturated fatty acids (PUFA)—the building blocks of lipid peroxide [5]. There are concerns about the potential health dangers and toxicity linked to the use of synthetic antioxidants [6]. The use of synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food items is restricted because of worries about their possible carcinogenic effects, as stated in [7]. Xanthophylls, carotenes, flavonoids, anthocyanins, ligans, and stilbenes are carotenoids; vitamins E and C are the principal components of natural antioxidants obtained from plant sources, according to [8-9]. *Crotalaria ramosissima*, known as Sunn or Sunn hemp, is a tropical Asian plant of the legume family (Fabaceae). *Crotalaria ramosissima* is thought to have come from India, where it has been grown since ancient times. It is now grown all over the tropics and subtropics. India is home to about 75 kinds of *Crotalaria* (The Wealth of India 1950). In terms of business, *C. juncea* is the most important one. In Indian medicine, some species of Crotalaria are used to treat certain illnesses [10]. The leaves of *C. verrucosa* and *C. retusa* can help treat scabies and impetigo, and the roots of *C. albida* and *C. trifoliastrum* can help empty the intestines. People use the roots of *C. prostrata* [11] to treat diarrhea in babies and children. To find out more about the health benefits of *Crotalaria ramosissima* leaf in terms of primary phytochemicals, secondary metabolites, antioxidants, and reducing inflammation, this study was done.

MATERIALS AND METHODS

Quantification of secondary metabolites

Quantification of total phenolics

The determination of the total phenolic content was conducted using the methodology outlined [12]. The measurement of absorbance was conducted at a wavelength of 725 nm using the reagent blank. Gallic acid equivalents were obtained from the triplicate analysis.

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Citation: Gokila T, Joseph S. 2024. Secondary metabolites, *in vitro* antioxidant and anti-inflammatory activity of *Crotalaria ramosissima* Roxb. leaves. *Res. Jr. Agril. Sci.* 15(3): 789-795.

Quantification of total tannins

The tannins were extracted from the same extract after being subjected to PVPP treatment. The only phenolic compounds that are present in this supernatant are simple phenolics; tannins would have been precipitated along with the PVPP. The non-tannin phenolics that were present in the supernatant were measured by employing the method described above [13].

Tannins = Total phenolics - Non tannin phenolics

Quantification of total flavonoids

The procedure that was provided [14] was utilized in order to determine the flavonoid content of the extracts of *C. ramosissima*. At a wavelength of 510 nm, spectrophotometry revealed the pink color of flavonoids. According to the calibration curve, the total flavonoid content was calculated using the Quercetin Equivalents (mg/g) unit of measurement.

In vitro antioxidant activity

DPPH scavenging activity

The absorbance at 517 nm was determined for the samples, control, and methanol blank. IC50 values represent the concentration of a sample required to stop 50% of the activity of DPPH [15].

ABTS radical cation scavenging activity

The ABTS radical cation scavenging test, as described [16], was employed to measure the overall antioxidant activity. The measurement of absorbance was conducted at a wavelength of 734 nm for both samples and standards, namely BHT and Rutin, in comparison to the ethanol blank. The findings were quantified by determining the quantity of Trolox with equal antioxidant activity, which was then represented as µM/g for the sample extracts.

Ferric reducing antioxidant power (FRAP) assay

The antioxidant capabilities of several sample extracts were assessed using the methodology outlined [17]. The spectrophotometer was used to promptly measure the absorbance at a wavelength of 593 nm, relative to the reagent blank. The equivalent concentration was determined by calculating the concentration of antioxidant that resulted in an absorbance rise in the FRAP experiment, which is equal to the theoretical absorbance value of a 1 mM concentration of Fe (II) solution.

Superoxide radical scavenging activity

The experimental procedure relied on the ability of different extracts to hinder the synthesis of formazan by scavenging the superoxide radicals produced in the riboflavin–light–NBT system, as described [18]. The measurement of absorbance was conducted at a wavelength of 590 nm, using a blank sample, immediately following illumination. Determined the scavenging activity of superoxide anion generation using the given formula:

$$\text{Scavenging activity (\%)} = [(\text{Control OD} - \text{Sample OD}) / \text{Control OD}] \times 100$$

Phosphomolybdenum assay

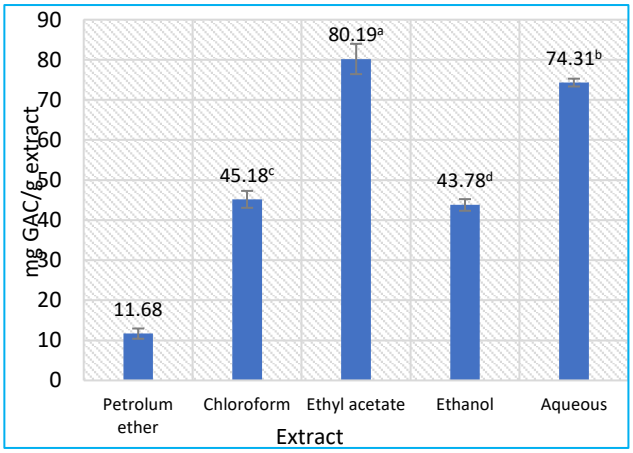
The determination of the antioxidant activity of the samples was conducted using the green phosphomolybdenum complex production technique, as described [19]. The absorbance of the combination was measured at a wavelength of 695 nm, relative to the reagent blank. The reference standard used in this study was the ascorbic acid equivalents (AAE) per gram of extract.

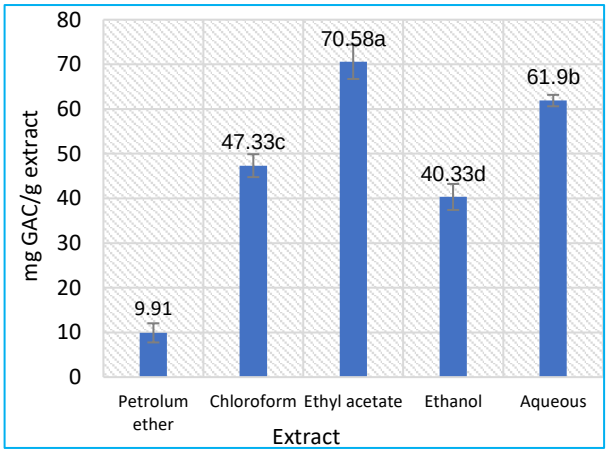
In vitro anti-inflammatory activity

Membrane stabilization method

According to [20], the sterilization of Alsever's solution involved the dissolution of a mixture consisting of 2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in distilled water. The supernatant haemoglobin was measured at a wavelength of 560 nm using spectrophotometry. Formula for determining the percentage of membrane stabilization:

$$\text{Percentage membrane stabilization inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100$$





Values are mean of triplicate determination (n=3) ± standard deviation, GAE - Gallic Acid Equivalent
Statistically significant at $p < 0.05$ where a > b > c > d

Fig 1 Total phenolics content of *Crotalaria ramosissima* leaf extracts

Fig 2 Total tannins content of *Crotalaria ramosissima* leaf extracts

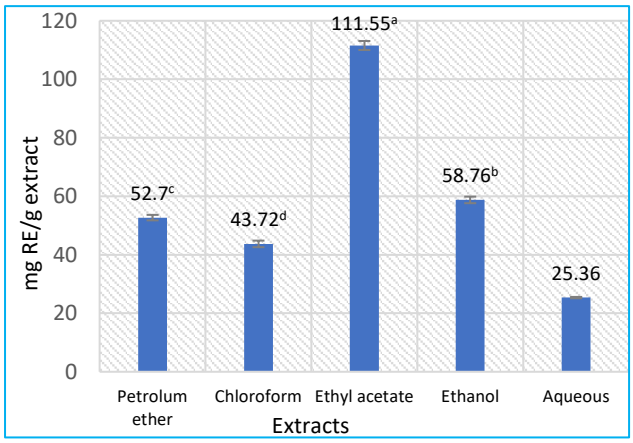
RESULTS AND DISCUSSION

Quantitative analysis of secondary metabolites

Determination of total phenolics and tannin contents

In this study, the total phenolics content is all the leaf extracts were estimated by Folin–Ciocalteu reagent method and the results are shown in (Fig 1-2). The total phenol content was expressed in Gallic Acid Equivalents (GAE). The highest

amount of total phenolic was obtained from ethyl acetate leaf extract of *Crotalaria ramosissima* (80.19 mg GAE/g extract) and followed by aqueous extract (74.31 mg GAE/g extract). The results clearly indicated that the *C. ramosissima* ethyl acetate extract contains the highest total phenolic content when compared to other extracts. The tannins were found to be higher in ethyl acetate extracts of the leaf (70.58 mg GAE/g extract) followed by aqueous, chloroform, ethanol, and petroleum ether extracts. Phenolics are aromatic compounds which protect the plants against stress and highly contribute to the antioxidant capacity [21-22]. Phenolic compounds are relatively stable phenoxyl radicals, disrupting chain oxidation reactions in cellular components [23]. These play a specific role in scavenging of free radicals [24]. These compounds highly contribute to the antioxidant capacity which helps in maintaining health and protection from severe disorders [25]. Tannins are bitter tasting organic compounds that have been reported to have strong astringent properties such as antimicrobial, anti-inflammatory and antioxidant activity causing protein precipitation [26].



Values are mean of triplicate determination (n=3) ± standard deviation, GAE - Gallic Acid Equivalent
Statistically significant at $p < 0.05$ where a > b > c > d

Fig 3 Total flavonoid content of *Crotalaria ramosissima* leaf extracts

Determination of flavonoid contents

The flavonoid content in different extract of *C. ramosissima* leaves were analysed (Fig 3). The total flavonoid content was expressed in Rutin equivalents (RE). Among all the extracts, the ethyl acetate extracts (111.55 mg RE/g) were found to have appreciable amount of flavonoid contents. The results revealed that the ethyl acetate extract contains the highest total flavonoid content. Flavonoids are most common and widely distributed group of polyphenolic compounds. These play a major role as antioxidants derivative in scavenging the free radicals and chelate trace elements [27-30]. Flavonoids play a crutial role in cellular level as it regulates cell cycle [29]. It is very much important and useful in the treatment of cardiovascular diseases as it possesses potent antioxidant and anti-inflammatory properties [30].

In vitro antioxidant activity

DPPH radical scavenging activity

The DPPH radical scavenging activity of various extracts of *C. ramosissima* leaf are shown in (Fig 4). The IC50 value of all the extracts was calculated, indicating the concentration of the extracts needed to eliminate 50% of DPPH free radical. A low IC_{50} value indicates a significant antioxidant capacity. The ethyl acetate extract exhibited an IC_{50} value of 34.46 µg/mL. In comparison to other extracts, including ethanol, aqueous, chloroform, and petroleum ether, the ethyl acetate extract shown promising antioxidant activity in the leaf. The leaf sample under investigation has significant free radical scavenging activity, as evidenced by its comparison with the natural antioxidant rutin (7.93 µg/mL) and synthetic antioxidant BHT (6.35 µg/mL). DPPH is an organic chemical compound consisting of a stable free radical molecule that has been widely employed in the study of antioxidant properties due to its resistance to degradation in water, methanol, and ethanol. According to [31], DPPH free radicals have the ability to readily acquire an electron or hydrogen from antioxidant molecules, resulting in the formation of a stable diamagnetic molecule. According to the findings of [32], the plant extract has the capacity to decrease the presence of the stable DPPH radical, as evidenced by a noticeable alteration in color from purple to yellow.

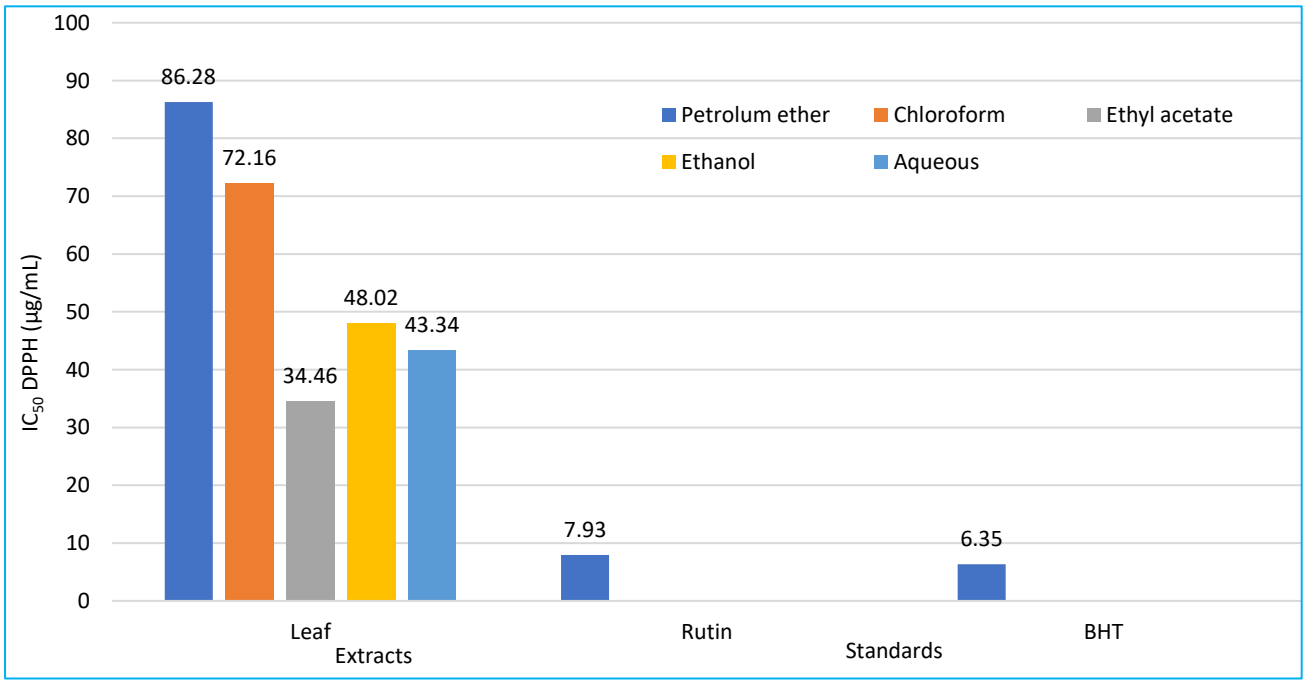
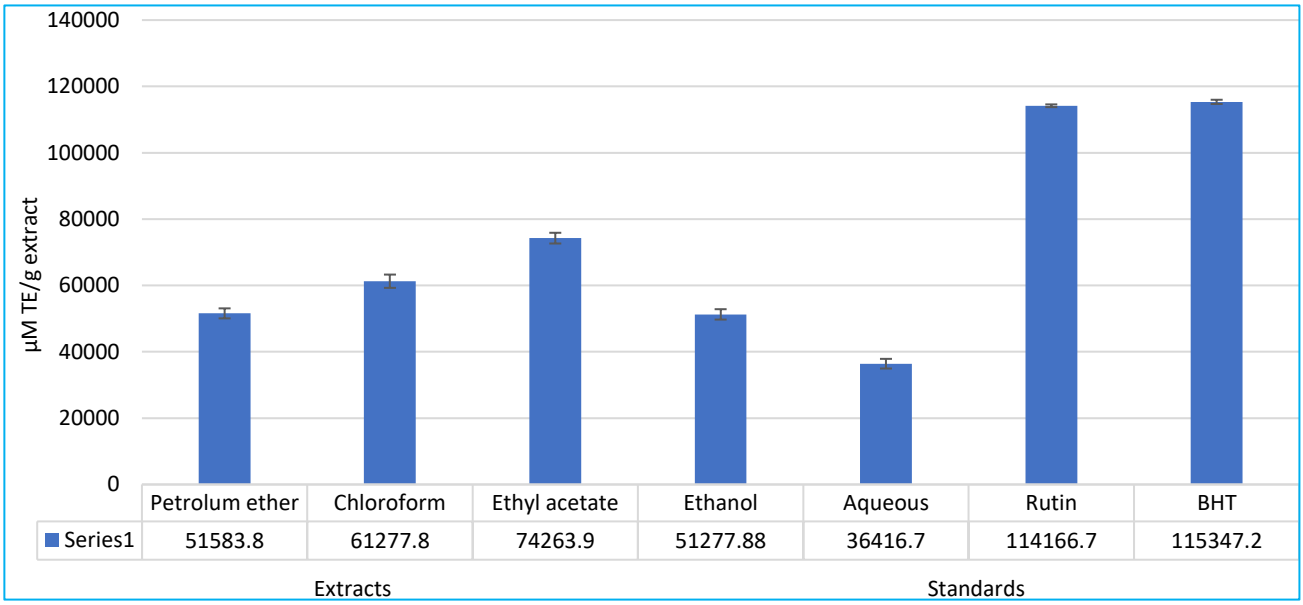


Fig 4 DPPH radical scavenging activity of *Crotalaria ramosissima* leaf extracts

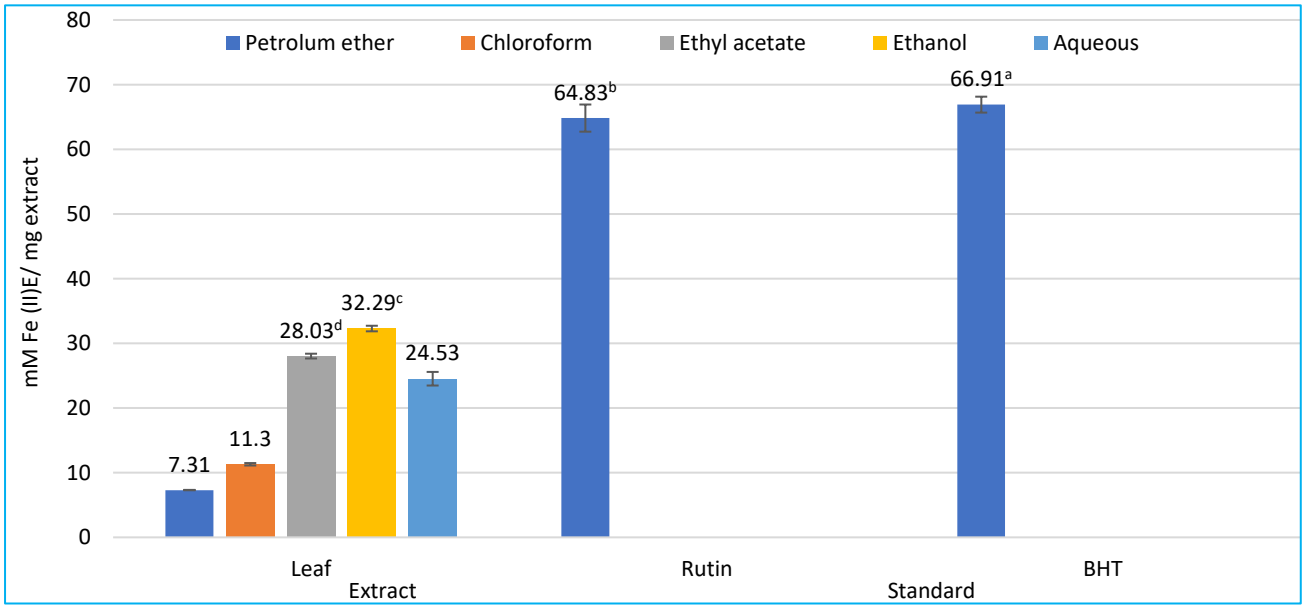
ABTS cation radical scavenging activity

The TEAC (Trolox Equivalents Antioxidant Capacity) was quantified using the enhanced ABTS+ radical decolorization test. The findings were quantitatively represented as µM Trolox Equivalents per gram of extract. Figure 5 displays the outcomes of the ABTS cation radical scavenging activities of *C. ramosissima* leaf. The ethyl acetate extracts exhibited a much greater radical scavenging activity of 74263.9 µM TE/g compared to the chloroform, petroleum ether, ethanol, and water extracts. The concentration of the natural antioxidant rutin (114166.7 µM TE/g sample) and the synthetic antioxidant BHT was determined to be 115347.2 µM TE/g extract. According to [33-34], the ABTS radical exhibits solubility in both aqueous and organic solvents, and its effectiveness is not influenced by ionic strength. Consequently, it may be employed to assess the antioxidant capacity of hydrophilic and lipophilic substances present in the chosen test materials. Phenolics may be effectively assessed for their antioxidant ability using ABTS radicals, as these radicals possess relatively lower redox potentials. The thermodynamic characteristic of the ABTS radical allows for the reactivity of several phenolic compounds [35]. According to [36], it has been observed that high molecular weight phenolic compounds exhibit a greater capacity to counteract the effects of free radicals (ABTS). The efficiency of these compounds is influenced by factors such as molecular weight, the number of aromatic rings, and the type of substitution of hydroxyl groups, rather than the particular groups involved.



Values are mean of triplicate determination (n=3) ± standard deviation, TE - Trolox Equivalents

Fig 5 ABTS cation redical scavenging activity of *Crotalaria ramosissima* leaf Extracts (µM TE/g extract)



Values are mean of triplicate determination (n=3) ± standard deviation; Statistically significant at $p < 0.05$ where a > b > c > d

Fig 5 Ferric reducing antioxidant power (FRAP) assay of *Crotalaria ramosissima* leaf extracts

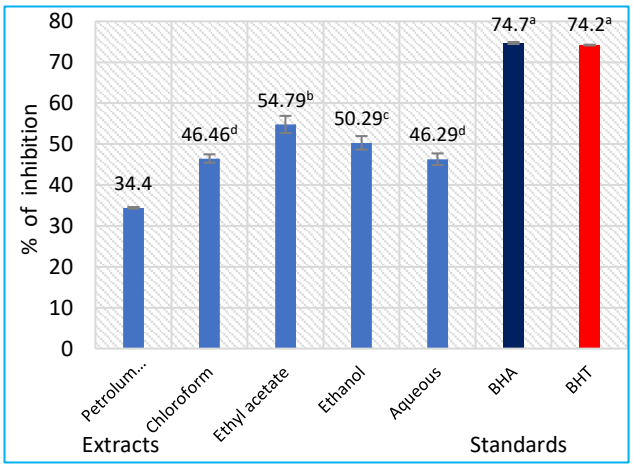
Ferric reducing antioxidant power (FRAP) assay

The test, which is both straightforward and dependable, quantifies the ability of an antioxidant to reduce a ferric 2, 4, 6-tripyridyl-S-triazine (Fe (III)-TPTZ) complex. This reaction results in the formation of a colored ferrous 2, 4, 6-tripyridyl-S-triazine (Fe (II))-TPTZ) complex when a reductant is used at a low pH. The monitoring wavelength for this compound is 593 nm. A greater level of absorption power is indicative of an

increased capacity for ferric reduction. The findings indicated, as seen in (Fig 5), that the ethanol extract of *Crotalaria ramosissima* leaf exhibited a significantly greater ferric reducing capacity (32.29 mM Fe (II)/mg extract) compared to the ethyl acetate extract (28.03 mM Fe (II)/mg extract). The values of the synthetic antioxidant BHT (66.91 mM Fe (II)/mg extract) and the natural antioxidant Rutin (64.83 mM Fe (II)/mg extract) were evaluated by comparison. The findings of the study indicate that the ethanol and ethyl acetate extracts derived from the leaf exhibit favorable reducing power activity. The FRAP test is widely regarded as a superior method for evaluating antioxidant potential due to its straightforward, efficient, and consistent nature. This is attributed to its ability to establish a strong correlation between the molar concentration of antioxidants and their capacity to reduce ferric ions [37]. The underlying mechanism of this process is predicated on the capacity of electron-donating antioxidants to undergo reduction in an acidic environment. Consequently, a ferric complex ($Fe^{3\pm}$ tripyridyltriazine) that lacks color undergoes a transformation into a ferrous complex ($Fe^{2\pm}$ tripyridyltriazine) that exhibits a blue hue. This transformation is quantified by measuring the maximum absorbance at a wavelength of 593 nm [38].

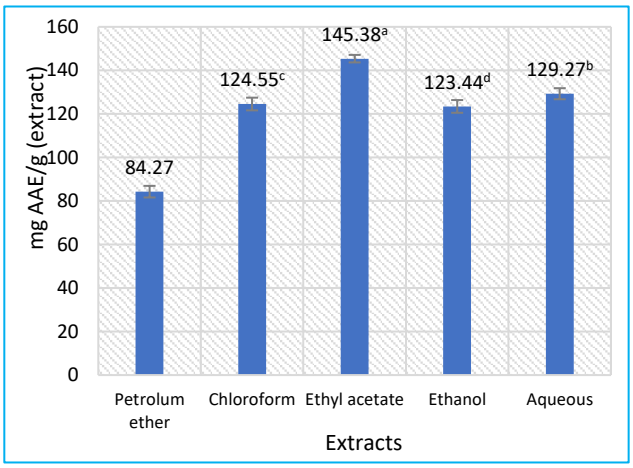
Superoxide radical scavenging activity

The superoxide radical is well recognized as a severe threat to cellular constituents due to its role as a precursor to more reactive oxygen species. The production of the superoxide radical in vivo leads to the synthesis of hydrogen peroxide (H_2O_2) through a dismutation process. (Fig 6) displays the outcomes of the superoxide anion scavenging activities of *Crotalaria ramosissima* leaf. The extracts shown high efficacy in scavenging superoxide radicals produced in an in vitro riboflavin-NBT-light system. The ethyl acetate extract exhibited a scavenging activity of 54.79%, whereas the ethanol extract had a scavenging activity of 50.29%. The free radical scavenging activity of the ethyl acetate and ethanol extracts was found to be comparable to that of BHT (74.7%) and BHA (74.2%). According to [39], the superoxide radical serves as a precursor for reactive oxygen species such as hydrogen peroxide, hydroxyl, and singlet oxygen. These species are recognized for their detrimental effects on cellular components. While superoxide radical anions do not have the ability to directly trigger lipid oxidation, they serve as powerful precursors to highly reactive species like hydroxyl radicals. Therefore, investigating the scavenging of these radicals has significant importance [40].



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

Fig 6 Superoxide radical scavenging activity of *Crotalaria ramosissima* leaf extract



Values are mean of triplicate determination (n=3) ± standard deviation AAE- Ascorbic acid equivalent, statistically significant at $p < 0.05$ where a > b > c > d

Fig 7 Phosphomolybdenum assay of *Crotalaria ramosissima* leaf extracts

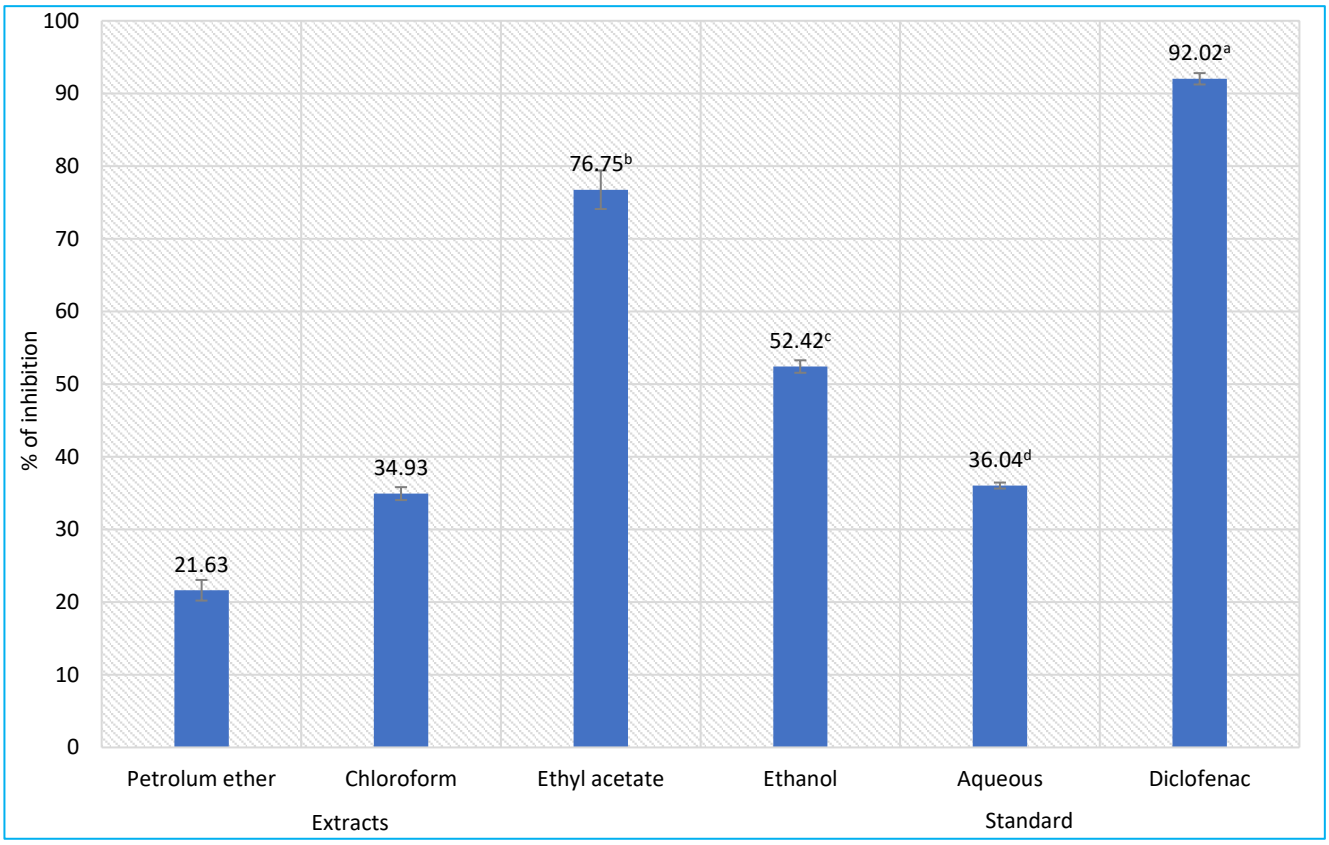
Phosphomolybdenum reduction assay

The analysis of the total antioxidant capacity of various extracts derived from selected leaves of *C. ramosissima* was conducted, and the findings are presented in (Fig 7). The ethyl acetate extract exhibited a higher antioxidant capacity (145 mg AAE/g extract) compared to the aqueous extract (129.27 mg AAE/g extract). Additionally, the remaining extracts exhibited favorable antioxidant activity. The primary basis of the phosphomolybdenum test is in the capacity of certain extracts to convert Mo (VI) to Mo (V) and subsequently generate a green phosphate/Mo (V) complex under acidic conditions [19].

In vitro anti-inflammatory activity

Membrane stabilization assay

The percentage inhibition of the extract was used to express the findings of the anti-inflammatory actions of *C. ramosissima* leaf, as seen in (Fig 8). In the current investigation, it was shown that the leaf ethyl acetate extracts exhibited a greater level of inhibitory activity (76.75%), with the ethanol extract following closely behind at 52.42%. The anti-inflammatory efficacy of the various extracts is ranked as follows: Diclofenac superior to ethyl acetate, followed by ethanol, aqueous, chloroform, and finally petroleum ether. In the context of inflammation, the process of lysosomal membrane lysis occurs, leading to the release of enzyme components that give rise to a range of illnesses [41-42] have reported that NSAIDs have the ability to either impede the release of lysosomal enzymes or enhance the stability of the lysosomal membrane. According to [43], the exposure of red blood cells (RBCs) to harmful substances such as hypotonic medium, heat, methyl salicylate, and phenyl hydrasine leads to the lysis of the RBC membrane, resulting in hemolysis and oxidation of hemoglobin. The investigation of the mechanism of anti-inflammatory effect involves examining the prevention of hypotonicity and heat-induced lysis in human red blood cell (RBC) membranes, which have resemblance to lysosomal membranes [44].



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

Fig 8 Membrane stabilization assay of *Crotalaria ramosissima* leaf extracts

CONCLUSION

In conclusion, *Crotalaria ramosissima* antioxidant activity makes it a promising dietary supplement to synthetic antioxidants. Thus, this study seeks to assess the value of traditional leaf for drug development. Bioactive molecule isolation and identification require more investigation. Antioxidant and anti-inflammatory medication mechanisms must be studied *in vivo*. These chemicals might be cost-effective and reliable therapy.

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