International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

Thin layer chromatography (TLC) and antioxidant activity of ethanolic extract of *Acalypha indica* linn

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International Journal of Science and Research Archive, 2025, 15(01), 1600-1606

Publication history: Received on 21 March 2025; revised on 27 April 2025; accepted on 30 April 2025

Article DOI: https://doi.org/10.30574/ijsra.2025.15.1.1265

Abstract

Acalypha indica is an important medicinal plant and traditionally used as wound healing, ulcer, arthritis, anti-inflammatory, anti-microbial, anti-diabetic and pneumonia. Thin Layer Chromatography (TLC) the present study showed separation of two deep violet colour spots with RF value 0.31 and 0.72 which may represent the presence of Alkaloids. Anti-oxidants are belived to play a very vital role in the body defense system against ROS. Plant based anti-oxidants with their prominence have gained tremendous worldwide interest now days. *Acalypha indica* used various diseases medication which have potential source as natural anti-oxidants. Anti-oxidant compounds may function as free radical scavengers, initiator of the complexes of pro-oxidant metals, reducing agents and quenchers of single oxygen formations.

Keywords: Acalypha indica; TLC; Anti-oxidant; Free radicals; Oxidative stress; Reactive oxygen stress

1. Introduction

Thin Layer Chromatography (TLC) method is an important analytical tool for the separation, identification and estimation of different classes of bioactive compounds [1]. *Acalypha indica* in particular have been found contain Phenolics, Tannin and Flavonoids [2]. These compounds have various biological properties, such as Anti-oxidant, anti-microbial, anti-ulcer, immunomodulatory, anti-diabetic, anti-inflammatory and hepato protective functions [3]. These phyto constituents give special characterstics and properties to plant. Therefore, the analysis of these bio active compounds in plants would help in determining various biological activity of plants [4].

Reactive Oxygen Species (ROS) is indispensable in many biological process mainly during cell differentiation, disease mechanisms, immunity etc [5]. Therefore ROS is produced in normal metabolic reaction and is maintained at physiological levels by several endogenous Anti-oxidant system. In addition exogenous ROS may be generated from environmental pollutants, excessive alcohol consumption, radiation exposure, viral and bacterial infections and others [6]. However, a condition in which ROS are excessively generated, oxidative stress is going be inevitable causing multiple cellular compartments, damage, cell injury or cell damage. The oxidative stress, triggered by the in balance between oxidants and antioxidants, eventually leads to many degenerative and chronic diseases in human [7].

Plants are the potential source of natural Anti-oxidants. Natural Anti-oxidants or phytochemical Anti-oxidants are the secondary metabolites of plants. Flavonoids, tocopherols, folic acid, carotenoids, ascorbic acid, cinnamic acid, benzoic acid, tocotrienols etc., are some of the Anti-oxidants produced by the plant for their substances. β- carotene, ascorbic acid and alpha tocopherol are the widely used Anti-oxidants [8]. Plant Anti-oxidants have been reported to prevent the occurrence of diabetes, cancer, and ageing. It can interfere with the oxidative process by reacting with free radicals, catalytic metals and also by acting as oxygen scavengers [9].

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Hence the search for natural Anti-oxidants is an alternative source which is of great interest among researchers. Plants are endowed with free radical scavenging molecules such as vitamins, terpenoids, phenolics, lignings, tannins, flavonoids, quinines, coumarins, alkaloids, amines and other metabolities, which are rich in Anti-oxidant activity [10, 11]. Studies have shown that many of these Anti-oxidant compounds possess anti-inflammatory, anti – athero sclerostic, anti tumour, anti-mutagenic, anti-carcinogenic, anti-diabetic, anti-bacterial and anti-viral activities [12,13].

2. Material and methods

2.1. Separation of Components by Thin Layer Chromatography (TLC)

2.1.1. Preparation of TLC Plates

25×10 cm glass plates were washed with distilled water followed by smearing with acetone. After drying the plates were placed on the template in row. The slurry of silica gel G prepared with glass distilled water in the ration 1:2 (w/v) was poured in the applicator. The glass plates were immediately coated with a layer of silica gel in 500 µm thickness. The coated plates were activated at 80^0C for 3h. Then the plates were stored in a plate chamber for further study. In that study, chloroform and methanol were used as solvents in the ratio of 96:4 [14].

2.2. Preparation of dragendorff's reagent

2.2.1. Solution A

0.6 g of Bismuth subnitrate was dissolved in 2 ml of concentrated hydrochloric acid and added 10 ml of distilled water.

2.2.2. Solution B

6 g of Potassium iodide was dissolved in 10 ml of water.

The solution A and B were mixed together with 7 ml of concentrated hydrochloric acid and 15 ml of water. The whole content was diluted to 400 ml with distilled water.

2.2.3. Solvent

Acetic acid : ethanol (1:3)

2.2.4. Loading of substances

The concentrated plant extract of 2.5 mg was loaded on the TLC plates just above 2 cm from the bottom using a capillary tube. The plates were reserved in a developing jar containing the solvent mixture. After the solvent front reached approximately 18 cm height, the plates were removed and allowed at room temperature for 30 min. Then the plates were observed by spraying with Dragendorff's reagent and recorded the R_f value of visualized spots.

2.3. Analysis of i*n vitro* antioxidant potential of *Acalypha indica*

2.3.1. Ferric reducing / antioxidant power (frap) assay

FRAP (900µl) reagent, prepared freshly and incubated at 37^0C, was mixed with 90 µl of distilled water and 30 µl of test sample. The test sample and reagent blank were incubated at 37^0C for 30 min. in a water bath. The final dilution of the test sample in the reaction mixture was 1:34. The FRAP reagent contained 2.5 ml of 20 mmol / 1,2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mmol / 1 HCL plus 205 ml of 20 mmol / 1 $Fe_3C1.6H_2O$ and 25 ml of 0.8 mol. of acetate buffer (pH 3.6). At the end of incubation, the absorbance reading were taken immediately at 593 nm using a spectrophotometer. The values are expressed as mmol Fe (II) mg^{-1} extract [15].

2.3.2. DPPH radical scavenging assay

The antioxidant activity of the extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The sample extract at various concentrations (100-500µg) was taken and the volume was adjusted to 100µl with methanol. 5 ml of 0.1 mM methanol solution of DPPH was added and allowed to stand for 20 min. at 27^0C. The absorbance of the sample was measured at 517 nm and the percentage radical scavenging activity of the sample was calculated as follows.

% DPPH radical scavenging = (OD of control – OD of sample / OD of control) × 100 activity

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration [16].

Tannic acid was used as a standard.

2.3.3. Hydroxyl radical scavenging assay

About 100-500 µg of solvent extract was added with 1 ml of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%) and 1 ml of Dimethyl Sulfoxide (DMSO) (0.85% v/v in 0.1M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90^{0}C for 15 min. in a water bath. After incubation, the reaction was terminated by the addition of 1 ml of ice-cold TCA (17.5% w/v). Three ml of Nash reagent (75g of ammonium acetate, 3 ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and raised the volume to 1L with distilled water) was added and left at room temperature for 15 min. The intensity of the colour formed was measured at 412 nm against reagent blank [17].

The % hydroxyl radical scavenging activity was calculated as follows

$$\% \text{ HRSA} = (\text{OD of control} - \text{OD of sample} / \text{OD of control}) \times 100$$

Mannitol, a classical OH scavenger was used as a positive control.

2.3.4. Superoxide radical scavenging assay

The assay was based on the capacity of the extract to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 ml reaction mixture contained 50mM sodium phosphate buffer (Ph 7.6), 20 mg riboflavin, 12M EDTA, 0.1 mg NBT and various concentrations (100-500 µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination, the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. The percentage inhibition of superoxide anion generation was calculated as follows:

$$\% \text{ inhibition} = (\text{OD of control} - \text{OD of sample} / \text{OD of control}) \times 100$$

Quercertin was used as a positive control [18].

2.3.5. Nitric oxide radical scavenging assay

3 ml of 10Mm sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) was mixed with different concentrations (100-500 µg / ml) of extract and incubated at room temperature for 15 min. After incubation time, 0.5 ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was read at 546 nm [19].

% NO radical scavenging = (OD of control – OD of sample / OD of control × 100 activity. Curcumin was used as a standard.

3. Results and discussion

Separation of phytochemicals fractions from the ethanolic extract of *Acalypha indica* was presented in Table 1 and Fig 1. The fractions were separated in the form of two deep violet colour spots with R_F values 0.31 and 0.72 respectively.

The antioxidant potential of ethanolic extract of *Acalypha indica* was evaluated by measuring the *in vitro* free radical scavenging activity. The ferric reducing antioxidant activity of ethanolic extract of *Acalypha indica* was analysed and the result showed that the selected plant extract exhibited a ferric reducing activity of 186.05±2.95 mmol (Fe II)/mg extract.

The results on DPPH radical scavenging activity are given in Table 2.In the present study, the DPPH radical scavenging activity has been increasing gradually from 100µg/ml concentration of ethanolic extract of Acalypha indica. The selected plant extract exhibited a maximum DPPH radical scavenging activity of 52.37% at 500µg/ml and the lower concentration (100µg/ml) showed a moderate antioxidant activity of 10.04%. The IC50 value of ethanolic extract of *Acalypha indica* was found as 476.19 and 13.1 for the standard Tannic acid. While comparing with the standard Tannic acid, the IC50 value of ethanolic extract of *Acalypha indica* was increased.

The results of hydroxyl radical scavenging activity of ethanolic extract of *Acalypha indica* are depicted in Table 3. The ethanolic extract of *Acalypha indica* exhibitied a maximum hydroxyl radical scavenging activity of 42.16% at 500µg/ml and a minimum activity of 8.92% at 100 µg/ml and the IC_{50} value of ethanolic extract of *Acalypha indica* was found as 574.71 when compared with 38.3 of standard Mannitol.

The superoxide radical scavenging activity of ethanolic extract of *Acalypha indicais* exhibited in Table 4. The results showed a dose dependent activity. The percentage scavenging activity of superoxide radical increases with increasing concentration of ethanolic extract of *Acalypha indica.* Out of the five concentrations tested for superoxide radical scavenging activity, higher concentrations (400 µg/ml and 500 µg/ml) of ethanolic extract of *Acalypha indica* demonstrated good radical scavenging activity, while 200 and 300µg/ml concentrations showed slightly better inhibition than 100µg/ml concentration. The IC_{50} value of the ethanolic extract of *Acalypha indica* was recorded as 568.18 which seems to be higher than that of IC_{50} value of the standard Quercetin(52.1).

The nitric oxide radical scavenging activity of ethanolic extract of *Acalypha indica* is presented in Table 5. Ethanolic extract of *Acalypha indica* exhibited consistent increase in scavenging activity of nitric oxide radicals with an increasing concentration and exhibited a maximum activity of 56.87% at 500 µg/ml and a minimum activity of 11.40% at 100 µg/ml concentration. The IC_{50} value of ethanolic extract of *Acalypha indica* was arrived as 427.35 which seems to be higher than the IC_{50} value (43.1) of the standard Curcumin.

From the results of *in vitro* Anti-oxidant and free radical scavenging activity of ethanolic extract of *Acalypha indica* obtained in the present study proved that *Acalypha indica* has significant Anti-oxidant and free radical scavenging activity, which might be attributed to its flavonoids, phenolic contents and other phytochemical constituents. Hence, the selected plant extract may be used as therapeutic agent in preventing oxidative stress related degenerative diseases.

Plants have several pharmacological roles such as Anti-oxidant, anti-cancer, anti-microbial, anti-fungal and anti-parasitic. Plants have free radical scavenging molecules, including flavonoids, phenolics, anthocynins and vitamins, which show Anti-oxidant like activity. It has been reported that the Anti-oxidant property of phytochemicals may be mitigated the oxidative stress in the biological system. Phytochemicals have been reduced the risk of many human diseases include cardio. Vascular disease, hepato-renal diseases, diabetes, cancers and neuro degenerative disorders. However, several herbal medicines are being derived directly or indirectly from plants that are considered as an important medicine currently in use for curing various human diseases.

Plant synthesize secondary metabolites which include alkaloids, flavonoids, saponins, terpenoids, steroids, glycosides, tannins, volatile oils etc. The therapeutic efficacy of plant is because of these secondary metabolites for curing many diseases. Phytochemicals are pharma cologically active compounds. The present study *Acalypha indica* reported to anti-inflammatory, anti-microbial, anti-ulcer, anti-arthritic, Anti-oxidant, anti-cancer, and anti-diabetic activity. It has been observed that numerous plants have pharmacological effects due to the presence of metabolites.

Table 1 Separation of phytocomponents of *Acalypha indica* by TLC

Fractions/spot	**Colour of the spot**	**R_f value of the spot**
Spot-I	Deep violet	0.31
Spot-II	Deep violet	0.72

Figure 1 TLC analysis of phytocomponents of *Acalypha indica*

Table 2 DPPH radical scavenging activity of ethanolic extract of *Acalypha indica*

Sample	**Concentration (µg/ml)**	**Percentage activity**	**IC_{50}**
Ethanolic extract of *Acalyphaindica*	100	10.04 ± 0.729	
	200	21.04 ± 0.317	
	300	33.10 ± 0.449	476.19
	400	41.88 ± 1.421	
	500	52.37 ± 0.400	
Tannic acid	2	16.09 ± 2.6	
	4	28.84 ± 0.7	
	6	33.26 ± 1.2	13.1
	8	36.87 ± 0.7	
	10	41.61 ± 1.67	

Values are mean of triplicate determination; ± standard deviation .

Table 3 Hydroxyl radical scavenging activity of ethanolic extract of *Acalypha indica*

Sample	**Concentration (µg/ml)**	**Percentage activity**	**IC_{50}**
Ethanolic extract of *Acalyphaindica*	100	8.92 ± 0.308	
	200	17.45 ± 0.178	
	300	28.27 ± 0.122	574.71
	400	35.74 ± 0.216	
	500	42.16 ± 0.394	
Mannitol	10	16.03 ± 0.1	
	20	18.11 ± 1.1	

	30	33.11 ± 1.21	38.3
	40	55.12 ± 1.1	
	50	67.55 ± 1.2	

Values are mean of triplicate determination ;± = Standard deviation

Table 4 Superoxide radical scavenging activity of ethanolic extract of Acalypha indica

Sample	**Concentration (µg/ml)**	**Percentage activity**	**IC_{50}**
Ethanolic extract of *Acalyphaindica*	100	8.96 ± 0.394	
	200	19.27 ± 0.333	
	300	28.26 ± 0.658	568.18
	400	35.66 ± 0.441	
	500	42.62 ± 0.500	
Quercetin	10	9.1 ± 0.1	
	20	18.2 ± 0.11	
	30	22.1 ± 0.21	52.1
	40	35.2 ± 0.13	
	50	48.3 ± 0.14	

Values are mean of triplicate determination; ± =Standard deviation

Table 5 Nitric oxide radical scavenging activity of ethanolic extract of Acalypha indica

Sample	**Concentration (µg/ml)**	**Percentage activity**	**IC_{50}**
Ethanolic extract of *Acalyphaindica*	100	11.40 ± 0.147	
	200	23.98 ± 0.564	
	300	36.63 ± 0.250	427.35
	400	47.67 ± 0.306	
	500	56.87 ± 0.278	
Curcumin	10	15.1 ± 01	
	20	25.2 ± 0.2	
	30	30.3 ± 1.1	43.1
	40	45.4 ± 1.21	
	50	60.4 ± 1.3	

Values are mean of triplicate determination; ± = Standard deviation

4. Conclusion

Phytochemical analysis of Acalypha indica showed a very rich source of tannins, saponins, terpenoids, flavonoids, phenols and glycosides. The presently investigated Acalypha indica Phytoconstituents which may be responsible for its therapeutic ability as an antioxidant, antimicrobial, anti inflammatory, anti ulcer, and anti arthritic activity. Plants with natural products exhibit phaPharmacological activity, play an important role in life threatening conditions and for the establishment of several pharmaceutical industries.

Compliance with ethical standards

Disclosure of conflict of interest Authors declare that they have no conflict of interest.

No conflict of interest to be disclosed.

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