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Evaluation of In-Vitro Anti-inflammatory and Antioxidant Activity of *Amaranthus Spinosus Linn*

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ABSTRACT
Advancements in science and technology are leading to more effective anti- inflammatory treatments. Identifying new plant-derived pharmacological molecules can provide beneficial phytoconstituents that reduce inflammation with fewer side effects. Inflammation is considered to be a crucial factor in development of chronic diseases, eight of which were listed among the top ten causes of death worldwide in the World Health Organization. There are the traditional claims on Amaranthus spinosus that be used for anti-inflammatory and antioxidants. In this research we assessed the anti-inflammatory and antioxidant properties of amaranthus by using the different methods such as 2,2-Diphenyl-1-picryl hydrazyl radical scavenging ability assay and Hydrogen peroxide scavenging assay for antioxidant activity and for anti-inflammatory activity we studied on Inhibition of Bovine Serum Albumin(BSA) denaturation, Protease inhibition assay, Hypotonicity-induced haemolysis, Heat induced haemolysis. Also we studied about the phytochemical constituent of amaranthus spinosus linn. On the basis of the results, we conclude that the methanolic extract of *Amaranthus spinosus* linn considered as a sole source of anti-inflammatory and antioxidant. However, the isolation of active constituents from this *Amaranthusspinosus* linn species and its action responsible for itsanti-inflammatoryand antioxidanteffect is useful for the treatment of the same in the future.

1. INTRODUCTION:

Inflammation is the local response of living mammalian tissues to injury from any agent.It is a body defense reaction in order to eliminate or limit the spread of injurious agents, followed by removal of the necrosed cells and tissues.[1] Inflammation is one of the common events in majority of acute as well as chronic diseases and represents a cheif cause of morbidity in today‟s era of modern lifestyle. Inflammation leads to development of rheumatoid arthritis, diabetes, cancer, alzheimer‟s disease and atherosclerosis. Inflammation involves a complex network of many mediators, a variety of cells and

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execution of multiple pathways.[2] Inflammation is defensive response of an organism against invasion by the foreign bodieslike bacteria, viruses and parasities. An acute inflammatory response is manifested as redness, pain, swelling, heat and loss of function. Chronic inflammation is linked with various steps of tumorigenesis and recognized as risk factor for occurence of different types of cancers. Treatment of chronic inflammatory diseases like rheumatoid arthritis and inflammatory bowel diseases is still a challenge due to lack of safe and effective drugs.[3] Inflammatory diseases, including different types of rheumatic diseases, are a major health care problem worldwide.

Advancements in science and technology are leading to more effective anti- inflammatory treatments. Identifying new plant-derived pharmacological molecules can provide beneficial phytoconstituents that reduce inflammation with fewer side effects.[4] Inflammation is considered to be a crucial factor in development of chronic diseases, eight of which were listed among the top ten causes of death worldwide in the World Health Organization‟s World Health Statistics 2019. Morever, traditional drugs for inflammation are often linked to undesirable side effects. As alternatives to traditional anti-inflammatory drugs, plant-derived bioactives have been shown to be effective interventions against various chronic diseases, including Alzheimer‟s disease, cardiovascular disease and cancer.[5] The World Health Organization (WHO) estimates that approximately 65% of the world‟s population incorporates traditional medicine (ethnobotanical uses) into medical care. Ethnobotanical studies over the years have allowed the association of highly diversified plants with biological activities, from observation, description, and experimental research, which hasgreatly contributed to the discovery of natural products with biological action. Inflammation is considered to be a crucial factor in development of chronic diseases, eight of which were listed among the top ten causes of death worldwide in the World Health Organization‟s World Health Statistics 2019. Morever, traditional drugs for inflammation are often linked to undesirable side effects. As alternatives to traditional anti-inflammatory drugs, plant-derived bioactives have been shown to be effective interventions against various chronic diseases, including Alzheimer‟s disease, cardiovascular disease and cancer.[13] The World Health Organization (WHO) estimates that approximately 65% of the world‟s population incorporates traditional medicine (ethnobotanical uses) into medical care. Ethnobotanical studies over the years have allowed the association of highly diversified plants with biological activities, from observation, description, and experimental research, which hasgreatly contributed to the discovery of natural products with biological action. Finding a safe and effective drug to control inflammation has been challenge and therefore, many in vitro and in vivo (using animal models) have been developed for the evaluation of drugs having anti-inflammatoryproperties. In thepast few years, plant derived molecules known as phytochemicals or phytoconstituents or natural products appear to be a major source of drugs and being evaluated as a drug candidate for anti- inflammatory actions.[2]

Upon literature survey, we found one such plant that is*Amaranthus spinosus* linn belonging to family *Amaranthaceae* is widely distributed in Asian countries, from India, Japan and Indonesia. The plant is well known in Ayurveda systems. It has been used for the treatment of different ailments such as cancer, diabetes, cough, bronchitis, dyspnea, burning sensation, leprosy, piles, skin diseases, edema, snake bites, nausea, urinary calculi, and urinary tract disorders, abdominal pain,arthritis. Recent invivo studies have indicated its use as anti-diabetic, antiinflammatory, antioxidant, antihyperlipidemic, antimicrobial, anthelmintic, antipyretic, antitumor, antifertility, spasmolytic and bronchodilator properties.The aerial part of the plant contains a large number of phytochemicals mainly flavonoids, steroids, triterpenoids,glycosides, reducing sugars, phenolic compounds, anthraquinone, amino acids,and saponins.[6]

Upon literature survey, we found one such plant that is*Amaranthus spinosus* linn belonging to family *Amaranthaceae* is widely distributed in Asian countries, from India, Japan and Indonesia. The plant is well known in Ayurveda systems. It has been used for the treatment of different ailments such as cancer, diabetes, cough, bronchitis, dyspnea, burning sensation, leprosy, piles, skin diseases, edema, snake bites, nausea, urinary calculi, and urinary tract disorders, abdominal pain,arthritis.

Literature survey, also revealed that *Amaranthus Spinosus* linn extract contains flavonoids, tannins, terpenoids, steroids, tannin, carbohydrates and other polyphenolic compounds. All these compounds are known possesses anti-inflammatory, anti-oxidant and other activities.[6] Also, there are traditional claims on *Amaranthus spinosus* linnto beused as an anti-inflammatory and antioxidant.

So, the present study is planned with the objective to evaluate the in-vitro anti- inflammatory and antioxidant activity of *Amaranthus spinosus* linn leaves extract to authenticate and reconfirm the traditional claims.

Journal of Molecular Science
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1. Materials and Methods

A. Phytochemical Analysis[7,8]

The physiochemical tests were carried out for the above mentioned plants extracts using the standard procedures to identify the components.

Test for Alkaloid

1.Mayer's Test: Take 5 ml of filtrate, 2-3 drops of Mayer's reagent (Potassium mercuric iodide solution) were added, this leads to formation of cream / yellow colored precipitate indicates the presence of alkaloids.

2.Wagner's Test: Take 1 ml of filtrate, add 3-5 drops of Wagne's reagent (Solution of Iodine in Potassium Iodide) and observed the formation of reddish brown precipitate or colouration shows the presence of alkaloids.

Test for Flavanoids

1.Shinoda Test (Magnesium hydrochloride reduction test): To the test solution add few fragments of magnesium ribbon and add concentrated hydrochloric acid drop wise, pink orred colour shows presence of flavanoid .
yellow colour which turns to colourless by the addition of few drops of dilute acetic acid indicate the presence of flavonoids.

Test for phenolic compounds

1.Ferric Chloride Test: To the test solution and add few drops of neutral 5% ferric chloride solution. A dark green colour indicates the presence of phenolic compounds.

2.Lead Acetate Test: To the test solution and add few drops of 10% lead acetate solution. White Precipitate indicates the presence of phenolic compound.

Test for Tannin

1.FerricChlorideTest: To the test solution, few drops of ferric chloride test reagent were added.Anintense green or bluecolour developed was taken as an evidence for the presence of tannins.

2.Lead Acetate Test: To the test solution, a few drops of 10% lead acetate were added, and formation of precipitate indicate the presence of tannins.

Test for Glycosides

1.Keller Killiani test: To 0.5 g of plant extract, add 0.4 ml of glacial acetic acid containing a trace amount of ferric chloride. Transfer to a small test tube; add carefully 0.5 ml of concentrated sulphuric acid along the sides of the test tube, blue colour appears in the acetic acid layer, indicate the presence ofglycosides.

2.Borntrager's Test (Anthraquinone Glycosides): 0.5 g of the plant extract was shaken with benzene and organic layer got separated and half of its own volume of 10% ammonia solution added. A pink, red or violet coloration in the ammonical phase indicated the presence of anthraquinoneglycosides.

Test for Amino Acid and Protein

1.Nimhydrin Test: Take 1 ml of test solution , add 2-5 drops of Ninhydrin solution and keep it in a boiling water bath for 1-2 minutes. Observe for the formation of purple colour.

Test for Carbohydrates

1. Molisch's Test: Treat the 1ml of test solution with few drops of molisch‟s reagent. Add 0.2 ml of concentrated sulphuric acid slowly along the sides of test tube, stand for 2-3 minutes. Observe the formation red or violet colour ring appears at the junction.

2.Fehling's Test: Equal volume of Fehling„s A (Copper sulphate in distilled water) and Fehling's B (Potassium tartarate and sodium hydroxidein distilled water) reagents are mixed and few drops of sample is a added and boiled ,a brick red precipitate of cuprous oxide forms, if reducing sugars are present.

Test for terpenoids

Salkowski Test: Take test solution , treat with 0.5 ml of conc. HCL shaken well and allow to stand for some time, formation of yellow coloured indicate the presence of terpenoids.

Test for steroids

Libermann-Burchard Test: Extract treated with few drops of acetic anhydride, boil and cool, concentrated sulphuric acid is added along the side of test tube, shows brown ring at the junction of two layers and the upper layer turns green which shows the presence of sterols and formation of deep red colour indicate the presence of triterpenoids.

Test for Saponin

Foam Test: A pinch of the dried powdered plant was added to 2-3 ml of distilled water. The mixture was shaken vigorously. Formation of foam indicates the presence of saponin.

B. Assessment of Antioxidant activity

1. 2, 2-Diphenyl-1-picryl hydrazyl radical scavenging ability assay (DPPH)[9,10]

Principle:

The in-vitro method using DPPH (2,2-diphenyl-1-picryl-hydrazyl) is an antioxidant assay based on scavenging of the DPPH by antioxidant. In its radical form, DPPH shows an active absorption at

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517 nm. Upon reaction of the antioxidant with DPPH, the DPPH accept hydrogen donar, and the solution loses its color from purple to pale yellow. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as

(DPPH)+(H-A) DPPH-H+(A)
Antioxidants react with DPPH and reduce it to DPPH-H and a consequence the absorbance decreases. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Procedure:
Solution of DPPH (0.1mm) in methanol was prepared by dissolving 1.9 mg of DPPH in100 ml methanol. The different concentrations (20,40,60,80 and 100 µg / mL) of extracts was taken in test tube.1 ml DPPH mixed with 1 ml of test solution and standard ascorbic acid separately. The mixture was incubated for 30 minutes in the dark at room temperature and the absorbance was measured at 517 nm. The degree of DPPH purple decolorization to DPPH yellow indicated the scavenging efficiency of the extract. Lower absorbance of the reaction mixture indicated higher free radical- scavenging activity.

The scavenging activity against DPPH was calculated using the equation:

DPPH scavenging activity(%)=Ac -At/Ac X 100
Where,
Ac is the absorbance of the control reaction (1ml of methanol with1ml of DPPH solution)
At is the absorbance of the test sample. The results were analyzed in triplicate.

2. Hydrogen peroxide scavenging assay (H_2O_2)[9,11]
Principle:
The working principle of hydrogen peroxide scavenging assay is the measurement of the antioxidant activity by the calculation of inhibitory activity of antioxidants against the oxidation of fluorescent probe by hydroxyl radicals. This radical is produced through the radical initiation process by the catalyzer hydrogen peroxide. The radicals bind the fluorescent probe, until the appearance of antioxidant, gradually preventing the oxidation.

Procedure:
A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM pH 7.4). The different concentration (20,40,60,80 and 100 µg/ml) of hydrogen peroxide is determined byabsorption at 230 nm using a spectrophotometer. 0.1 ml of sample added with 3.4 ml phosphate buffer and 0.6 ml of 40Mm hydrogen peroxide. Ascorbic acid is used as standard drug. After 10 min, measure the absorbance at 230 nm, against blank solution containing phosphate buffer without H_2O_2. The percentage of hydrogen peroxide scavenging is calculated as follows:

H_2O_2 scavenging activity (%) =Ac-At/AcX100
Where, Ac is the absorbance of control
At is the absorbance of test

C.Assessment of In-vitro Anti-inflammatory activity
1.Inhibition of Bovine Serum Albumin(BSA)denaturation[12,13]
Principle:
Protein denaturation is defined as a process where due to external factors such as heat, strong acid or strong base; an organic solvent or a concentrated inorganic salt causes the protein to denature that means the proteins tertiary structure and secondary structure is disoriented. Enzymes lose their activity since the substrates are able to no longer attach to the active site. Disruption of the electrostatic, hydrogen, hydrophobic, and disulphide bonds in protein structure occurs. In addition, a complex array of enzyme activation, mediator release, cell migration, tissue breakdown and repair occur, causing of protein to lose its molecular conformation and functions or become denatured. It is therefore deduced that, compounds which are able to prevent these change and inhibit thermally or heat induced protein denaturation, have potential therapeutic value as anti-inflammatory agents.

Procedure:
Test solution (0.5 ml) consists of 0.45 ml of BSA (5% w/v aqueous solution) and 0.05 ml of the test solution (100,200,300, 400 and 500 µg/ml). Control solution (0.5 ml) consists of 0.45 ml of BSA (5% w/v aqueous solution) and 0.05 ml of distilled water. Standard solution (0.5ml) consist of 0.45 ml of BSA (5%w/v aqueous solution)and 0.05 ml of diclofenac sodium (100,200,300,400 and 500 µg/ml).All the above solutions were adjusted to pH 6.3 using 1N Hydrochloric acid. The samples were incubated at 37°C for 20 minutes and the temperature was increased to keep the samples at 57°C for 3 minutes. After cooling, 2.5 ml of phosphate-buffered saline was added to the above solutions. The absorbance was measured using UV-visible spectrophotometer at660 nm. The percentage inhibition of protein denaturation was calculated by the formula:

% inhibition=(Abs of control-Abs of sample/Abs of control)X100

Journal of Molecular Science
Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 971-981
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2. Protease inhibition assay[13,14]

Principle:

Mucus is a protease inhibitor that is vital in preventing protease activity during homeostasis, inflammation, tissue damage, and the development of cancer. Protease inhibitors frequently possess intrinsic qualities, such as regulation of cytokine production, signal transmission, and tissue remodeling, in addition to their anti- protease action, which aids in the conclusion of the inflammatory process.

Procedure:

The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100,200,300,400 and 500µg/ml). The diclofenac sodium was used as standard drug in different concentration (100- 500 µg/ml). The mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The percentage inhibition of protease inhibitory activity was calculated by using formula:

% inhibition= (Abs of control-Abs of sample/Abs of control) X 100

3.Membrane stabilization [16,17]

Principle:

As the inflammation progresses, the cell lysis takes place releasing various lysosomal enzymes, including proteases resulting in tissue damage and inflammatory reactions. Damage of cell membrane loses permeability resulting in cell lysis. As the erythrocyte membrane is similar to that of lysosomal membrane, inhibition of red blood cell heamolysis may provide insight into the inflammatory process. Stabilization of these cell membranes may inhibit the lysis and subsequent release of the cytoplasmic content which turn, minimize the tissue damage and hence the inflammatory response. Membrane of human erythrocytes is analogous to membrane of lysosomes therefore membrane stabilization assay of erythrocytes is used as a measure to study anti-inflammatory activity

Procedure:

Preparation of Red Blood cells(RBCs) suspension:
2-3drops of blood wash three times with saline. Collect and dilute the RBC layer to make 10 % v/v using × 1 phosphate buffer saline (PBS).

1. Heat induced haemolysis[18,19]

The reaction mixture (2ml) consisted of 1ml test sample of different concentrations (100,200,300,400 and 500 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Diclofenac sodium (100-500µg/ml) was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56ºC for 30 min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The Percentage inhibition of Heat Induced Haemolysis was calculated as follows:

% inhibition= (Abs control-Abs sample/Abs control) X100

2. Hypotonicity-induced haemolysis[20]

Different concentration of extract (100,200,300,400 and 500µg/ml), reference sample, and control were separately mixed with 1 ml of phosphate buffer, 2 ml of hyposaline and 0.5 ml of HRBC suspension. Diclofenac sodium (100-500µg/ml) was used as a standard drug. All the assay mixtures were incubated at 37ºC for 30 minutes and centrifuged at 3000 rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by a spectrophotometer at 560 nm. The percentage haemolysis was estimated by assuming the haemolysis produced in the control as 100%.

% inhibition = (Abs control-Abs sample/Abs control)X100

Statistical Analysis:

The experiments were performed in triplicates and results were expressed as mean± standard deviation (SD). Test results were calculated in Microsoft Excel2007software.The values of in-vitro anti-inflammatory and antioxidant activity of the methanolic extract of *Amaranthus spinosus* linn were expressed as mean ± standard deviation (n=3) for each sample.[21]

RESULTS:

A) Phytochemical analysis

Table No.1: Phytochemical analysis of methanolic extract of *Amaranthus spinosus* linn

Sr.No	Phytochemical Constituent	Tests	Results
1.	Alkaloid	Mayer,,stest Wagner,,stest	++ +++
2.	Flavanoid	Shinoda test Alkaline reagent	- +++
3.	Phenol and Tannin	Leadacetate test FeCl3 test	++ -

Journal of Molecular Science
Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 971-981
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4.	Glycosides	Borntrager,,stestKeller-killiani test	- +++
5.	Amino acid and Protein	Ninhydrin test	+
6.	Steroids	Libermann test	++
7.	Terpenoids	Salkowski test	++
8.	Carbohydrates	Molisch,,stest Fehling test	++-
9.	Saponins	Foaming test	++

Note: (+) Presence of phytochemical constituent (-) Absence of phytochemical constituent
+++High, ++medium, +low

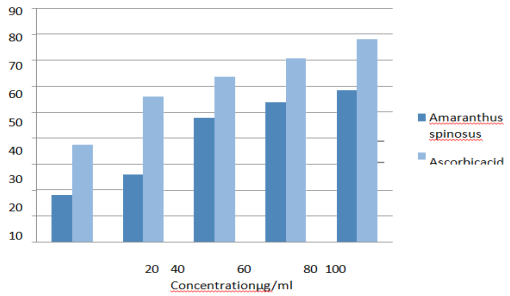
B) Assessment of in-vitro antioxidant activity

1. Effect of methanolic extract of *Amaranthus spinosus* linn on percent inhibition against DPPH assay

Table No.2:Effect of methanolic extract of *Amaranthus spinosus* linn on percent inhibition against DPPH assay

Sr.No	Conc. (µg/ml)	Control	Absorbance (Ascorbic acid)	Absorbance (MEAS)	% inhibition Ascorbic acid	% Inhibition (MEAS)
1	20	1.846	1.153	1.512	37.54 ± 0.48	18.09 ± 0.58
2	40	1.846	0.813	1.367	55.95 ± 0.80	25.94 ± 0.45
3	60	1.846	0.669	0.963	63.75 ± 0.74	47.83 ± 0.55
4	80	1.846	0.541	0.852	70.69 ± 0.43	53.84 ± 0.87
5	100	1.846	0.407	0.769	77.95 ± 0.63	58.34 ± 0.85

Values are expresses as mean ±S.D., n=3
IC_{50} value of *Amaranthus spinosus* linn was found t obe76.95µg/ml
IC_{50}value of Ascorbic acid was found to be 36.68µg/ml.



% inhibition

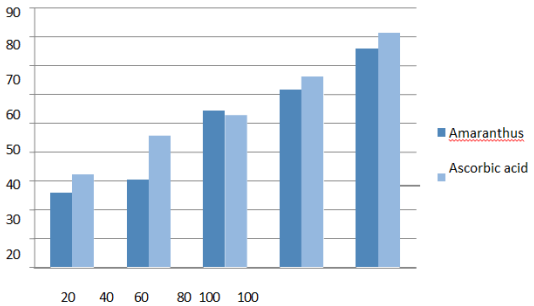
Fig no.1 :Effect of methanolic extract of *Amaranthus spinosus* linn on percent inhibition against DPPH Assay

2.Effect of methanolic extract of *Amaranthus spinosus*l linn on percent inhibition against H2O2 assay

TableNo.3: Effect of methanolic extract of *Amaranthus spinosus* linn on percent inhibition against H2O2 assay

Sr.No	Conc. (µg/ml)	Control	Absorbance (Ascorbic acid)	Absorbance (MEAS)	% inhibition (Ascorbic acid)	%inhibition (MEAS)
1	20	1.12	0.83	0.76	32.14± 0.49	25.89±0.80
2	40	1.12	0.78	0.61	45.53± 0.90	30.35 ±0.54
3	60	1.12	0.51	0.53	52.67 ± 0.87	54.46± 0.60
4	80	1.12	0.43	0.38	66.07 ±0.74	61.60± 0.41
5	100	1.12	0.27	0.21	81.25 ± 0.51	75.89± 0.34

Values are expresses as mean± S.D.,n=3
IC_{50}value of *Amaranthus spinosus* linn was found to be 60.57µg/ml
IC_{50}value ofAscorbic acid was found to be 50.75µg/ml.
% In



Figno.2: Effect of methanolic extract of *Amaranthus spinosus* linn on percent inhibition against H2O2 assay

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 971-981
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B) Assessment of Anti-inflammatory Activity

1. Effect of methanolic extract of *Amaranthus spinosus* linn on inhibition of BSA denaturation

Table No.4:Effect of methanolic extract of *Amaranthus spinosus* linn on inhibition of BSA denaturation

Sr.No	Conc. (µg/ml)	Control	Absorbance (Diclofenac sodium)	Absorbance (MEAS)	%inhibition (Diclofenac sodium)	%inhibition (MEAS)
1	100	1.43	0.97	1.09	32.16 ± 0.67	23.77± 0.44
2	200	1.43	0.73	0.89	48.95± 0.68	37.76 ± 0.68
3	300	1.43	0.54	0.65	63.57 ± 0.69	54.54± 0.33
4	400	1.43	0.39	0.46	74.28 ± 0.55	67.78 ±0.39
5	500	1.43	0.28	0.35	80.41 ± 0.69	75.52 ±0.43

Value are expresses as mean± S.D., n=3

IC50valueof*Amaranthusspinosus*linnwasfoundtobe287.14µg/ml

IC50value of diclofenac sodium was found be 220.49µg/ml.

% in

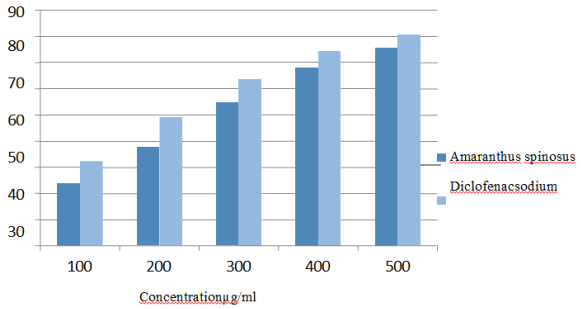

Fig no.3: Effect of methanolic extractof *Amaranthus spinosus* linn on inhibition of BSA denaturation

2. Effect of methanolic extract of *Amaranthus spinosus* linn on protease inhibition

Table No.5: Effect of methanolic extract of *Amaranthus spinosus* linn on protease inhibition

Sr. No	Conc. (µg/ml)	Control	Absorbance (Diclofenac sodium)	Absorbance (MEAS)	%inhibition (Diclofenac sodium)	%inhibition (MEAS)
1	100	1.923	1.236	1.403	35.72 ±0.45	27.04 ±0.42
2	200	1.923	0.940	1.206	51.11±0.72	37.28 ±0.83
3	300	1.923	0.786	0.960	59.12 ±0.42	49.92 ±0.72
4	400	1.923	0.630	0.730	67.23 ±0.58	62.03 ±0.76
5	500	1.923	0.530	0.660	72.43 ±0.44	65.67 ±0.52

Value are expresses as mean ±S.D., n=3

IC50 value of *Amaranthus spinosus* linn was found to be315.88µg/ml

IC50value of Diclofenac sodium was found to be 221.79µg/ml

3.Effect of methanolic extract of*Amaranthus spinosus* linn on heat induced haemolysis.

Table No.6: Effect of methanolic extract of*Amaranthus spinosus* linn on heat induced haemolysis.

Sr.No	Conc. (µg/ml)	Control	Absorbance (Diclofenac sodium)	Absorbance (MEAS)	%inhibition (Diclofenac sodium)	%inhibition (MEAS)
1	100	0.85	0.69	0.81	16.00 ±0.61	4.00 ±0.32
2	200	0.85	0.51	0.76	34.00 ±0.36	9.00 ±0.56
3	300	0.85	0.36	0.61	42.00 ±0.90	24.00 ±0.72
4	400	0.85	0.20	0.53	65.00±0.43	32.00 ±0.60
5	500	0.85	0.13	0.42	72.00 ±0.73	43.00 ±0.68

Value are expresses as mean±S.D.,n=3

IC50 value of *Amaranthus spinosus* linn was found to be573.26µg/ml

IC50value of Diclofenac sodiumwas found to be 329.37µg/ml.

4. Effect of methanolic extract *of Amaranthus spinosus* linn on hypotonicity induced haemolysis.

TableNo.7: Effect of methanolic extract *of Amaranthus spinosus* linn on hypotonicity induced haemolysis.

Sr.No	Conc. (µg/ml)	Control	Absorbance (Diclofenac sodium)	Absorbance (MEAS)	%inhibition (Diclofenac sodium)	%inhibition (MEAS)
1	100	1.22	1.13	1.19	7.37±0.18	2.45±0.31
2	200	1.22	1.07	1.14	12.29±0.36	6.55±0.50
3	300	1.22	0.92	1.02	24.59±0.32	18.03±0.43
4	400	1.22	0.71	0.88	41.80±1.57	27.86±0.59
5	500	1.22	0.43	0.67	64.75±0.43	45.08±0.32

Journal of Molecular Science
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Volume 35 Issue 3, Year of Publication 2025, Page 971-981
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DISCUSSION:

Phytochemical analysis of methanolic extract of *Amaranthus spinosus* linn revealed the presence of some secondary metabolites such as flavonoids, saponins, tannins, alkaloids, proteins, carbohydrates, phenol, glycosides, flavanoglycosides, betalin, amaranthin, steroids, stigmasterol, spinosterol, spinosides, quercitin. Due to the presence of flavonoids and polyphenolic compounds in *Amaranthus spinosus*linn shows the anti-inflammatory and antioxidant activity. Also, the presence of these phytoconstituents are responsible for different pharmacological activities.

In present study, we assessed the polyphenol-based antioxidant activity of *Amaranthus spinosus* linn. Plant-derived polyphenolic compounds showed ROS quenching property utilizing their structural chemistry, thus showing their antioxidant activity. The DPPH free radical scavenging assay was widely used to testthe antioxidant properties of plant extracts. Theantioxidant activitywas due to the presence of flavonoids and other phenolic contents. There were several mechanistic aspects that supported the antioxidant properties of phenolic acid (due to the presence of strong reaction of phenol moeties), the radical scavenging ability through donationofhydrogen atom was found to be a primary mechanism involved. The flavonoids also exhibited antioxidant effect by preventing the generation of ROS and scavenging them. Free radicals are known to be major factor in biological damages and have been used to evaluate the free radical scavenging activityof natural antioxidants, DPPH was aradical itselfwhich was purplein colour.It changes into a stable yellowcolourby reacting with an antioxidant, and the extent of reaction depends on the hydrogen, donating ability of the antioxidant. The reduction capability of the DPPH radical was determined by decrease in its absorbance at 517 nm. [22]

The antioxidant activity of methanolic extract of *Amaranthus spinosus* linn was analyzed by DPP free radical scavenging assay. Different concentrations (20,40,60,80 and 100 µg/ml) of methanolic extract of *Amaranthus spinosus* linn was evaluated against DPPH reagent via test tube method.The percent age inhibition of DPPH radical exhibited by different concentration was calculated and subsequently its IC_{50} was determined.The methanolic extract of*Amaranthus spinosus* linn shows good scavenging activity as compared to standard ascorbic acid.

Methanolic extract of *Amarathus spinosus* linn showed maximum inhibition at 100 µg/ml i.e.58.34% as compared to standard Ascorbic acid at 100 µg /ml i.e.81.25 %. Based on the results it observed that a percent of inhibition was directly proportional to the concentration. At 100 µg/ml it shows maximum percent inhibition that is 58.34%. The methanolic extract of *Amaranthus spinosus* linn scavenged 50 %DPPH free radical shows the moderate antioxidant activity at inhibitory concentration (IC_{50}) i.e.76.95 µg/ml as compared to standard Ascorbic acid was found to be 36.68µg/ml.

Oxidative stress is an important factor in the pathogenesis of many diseases. The *Amaranthus spinosus* linn plant extract with antioxidant activity have been shown the effect of oxidative stress induced by hydrogen peroxide.Generally, hydrogen peroxide is not much reactive, but inside a cell, itmayproduce hydroxyl radical. So, the elimination of H2O2 is very important to protect antioxidant in cell. [23] The antioxidant property of *amaranthus spinosus* linn plant is due to presence of secondary metabolites such as phenolic compounds, flavanoids, tannins. Antioxidant play an important role in scavenging free radicals and provide the protection against degenerative diseases. It has been reported that the phenolic compound present in the plant possess antioxidant activity due to the presence of hydroxyl group and they can act as potent hydrogen donars. Higher phenolic content and flavonoids in methanol extract is responsible for maximum reducing ability.[24]

In the present study, the methanol extracts of plants exhibited highest H2O2 activity due to the presence of polyphenols and flavonoids. The methanolic extract of *Amaranthus spinosus* linn was effective in inhibiting the freeradical scavenging activity at different concentrations (20,40,60,80, and 100µg/ml) as compared to standard ascorbic acid. The percentage inhibition was within range of 32.14% to 81.21%. At 100 µg/ml it shows maximum percent inhibition that is 81.21%. Test samples compared with standard Ascorbic acid. H2O2 absorbance was decreased as it oxidized by antioxidant species (plant extract, ascorbic acid). *Amaranthus spinosus* linn have ability to oxidize H2O2 by donating electrons, this ability of oxidizing was increased with inceased in concentration. The IC_{50}value of *Amaranthus spinosus* linn was found to be 60.57 µg/ml and Ascorbic acid was found to be 50.75 µg/ml. [23]

Inflammation is the response of living tissues to injury. It involves a complex array of enzyme activation, mediator release, and extravasation of fluid, cell migration, tissue breakdown and repair. The vitality of cells depends on the integrity of their membrane, exposure of RBC‟s to injurious substances such as hypotonic medium results in lysis of its membrane accompanied by haemolysis and the oxidation of haemoglobin. The haemolytic

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 971-981
DoI-10.004687/1000-9035.2025.131

effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will more susceptible to secondary damage through free radical induced lipid peroxidation. It is therefore expected that compounds with membrane- stabilizing properties, should offer significant protection of cell membrane against injurious substances. Compounds with membrane-stabilizing properties are well known for their ability to interfere with the release of phospholipase that trigger the formation of inflammatory mediators.

The methanolic extract of *Amaranthus spinosus* linn has shown significant membrane stabilizing property, which suggests that its anti-inflammatory activity observed in this study, it related to the inhibition of the release of phospholipases that trigger the formation of inflammatory mediators like prostaglandin and leukotriene etc through COX (cyclooxygenase) pathway.[25]

Bovine serum albumin denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well documented cause of inflammation. Auto-antigen production in inflammatory diseases, like rheumatoid arthritis, may able due to denaturation of protein in invivo condition.Alteration of electroelastic, hydrogen, hydrophobic nand disulphide bonding can be related to denaturation of protein. As part of the investigation on the mechanism of the anti-inflammation activity, ability of plant extract to inhibit protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation.

From this experiment we tested that methanolic extract of *Amaranthus spinosus* linn inhibited denaturation of protein and showed anti-inflammatory activity. The anti- inflammatory activity of amaranthus extract found due to the presenceoftherapeutically active flavonoids, alkaloids, glycosides and phenolic compounds.[26] Methanolic extract of *Amaranthus spinosus* linn leaves was able to inhibit Bovineserum albumin denaturation in a concentration dependent manner. The maximum percentage inhibition of methanolic extract of *Amaranthus spinosus* linn was 72.52% at 500 µg/ml as compared to standard diclofenac sodium was 80.41% at 500 µg/ml. The IC50 value of *Amaranthus spinosus* linn was found to be 287.14 µg/ml and diclofenac sodium was found be 220.49µg/ml. Protease such as trypsin and other serine protease are essential enzymes that mediate the hydrolytic breakdown of peptide bonds in proteins. They have been extensively studied with respect to their roles in inflammation, CVD, tissue remodelling, matrix destruction, auto immune arthritis and skin allergies. Therefore , therapeutic inhibition of these enzymes will be of novel contribution.

The result from the present study indicates that the methanolic extract of *Amaranthus spinosus* linn was able to inhibit protease in a concentration dependent manner. The percentage inhibition was within range of 27.04% to 65.67%.The *Amaranthus spinosus* linn compared with standard drug diclofenac sodium. As the concentration increases the percent inhibition increases. At 500 µg/ml it shows maximum percent inhibition that is 65.67% of *Amaranthus spinosus* linn.The maximum percent inhibition of standard Diclofenac sodium at 500 µg/ml i.e.72.43 %. The IC_{50} value of *Amaranthus spinosus* linn was found to be 315.88 µg/ml and standard Diclofenac sodium was found to be 221.79µg/ml.

Neutrophils are known to be a rich source of serine protease and are localized at lysosomes. It was previously reported that leukocytes protease play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by protease inhibitors [27,28].

The HRBC membrane stabilization has been used as a method to study the in vitro anti inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane.[29] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce a various disorders. The extra cellular activity of these enzymes said to be related to acute or chronic inflammation.[30] Thenon steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane.

The methanolic extract of *Amaranthus spinosus* linn was effective in inhibiting the heat induced haemolysis at different concentrations (100-500 µg/ml).[31] The percentage inhibition was within rangeof4.00%to 43.00%. Test samples compare with standard diclofenac sodium. As the concentration increases the percent inhibition increases. At 500 µg/ml heat induced haemolysis

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 971-981
DoI-10.004687/1000-9035.2025.131

shows highest percent inhibition thatis 43.00% respectively with IC_{50} value of *Amaranthus spinosus* linn was 573.26 µg/ml**.** However, the standard Diclofenac sodium shows highest percent inhibition i.e.72.00 % at 500 µg/ml with IC_{50} value of was found to be 329.37µg/ml.

The methanolic extract of *Amaranthus spinosus* linn the hypotonicity induced haemolysis shows maximum percent inhibition that is 45.08% at 500 µg/mland standard diclofenac sodium shows 64.75 % at 500 µg/ml. IC_{50} value of *Amarantus spinosus* linn was found to be 358.77 µg/ml and diclofenac sodium was found to be 256.11µg/ml. This effect could be due to the membrane stabilizing effect by preventing the release of lysosomal contents neutrophils at the site of inflammation.

CONCLUSION

In the present study, results indicate that the methanol extracts of *Amaranthus spinosus* linn possess anti-inflammatory andantioxidant properties. These activities may be due to the strong occurrence of polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, and phenols, glycosides, flavonoglycosides, spinoside, stigmasterol, spinosterol, quercitin, etc.

The results of present study showed that methanolic extract of *Amaranthus spinosus* linn leaves contain high amount of flavonoids, tannin and phenolic content and exhibited high antioxidant activity. Previous documentations showed that high scavenging activity is related to the presence of hydroxyl group in the phenolic compounds. As free radicals are highly involved in the pathogenesis of a lot of diseases so free radical scavengers can be preventive measure for those diseases. Thus observed radical scavenging activity of *Amaranthus spinosus* linn leaves extract can be explored for diseases prevention as well as nutraceutical application.

Inflammation is frequentlya keyelement in the pathological progression of organ disease. Three main pathways, NF-κB, MAPK, and JAK-STAT, play major roles in inflammation, and dysregulation of one or more of these pathways may lead to inflammation-associated disease. A better understanding of inflammatory response pathways and molecular mechanisms will undoubtedly contribute to improved prevention and treatment of inflammatory diseases.

The methanolic extract of *Amaranthus spinosus* linn were found to possess significant in vitro anti-inflammatory properties in several models such as denaturation of proteins, membrane stabilization and protease inhibitor. The pathogenesis of inflammatory diseases involves the over production of substance such as prostaglandin, arachidonic acid, and leukotrienes through cycloxygenase pathway. Purification of each bioactive compound is necessary and this purified form of the compound can be used which may show increased activity. This study gives on idea that the compound of the plant *Amaranthus spinosus* linn can be used as lead compound for designing a potent anti-inflammatory drug which can be used for treatment of various diseases occur due to chronic inflammation such as cancer, neurological disorder, aging and inflammation.

On the basis of the results, we conclude that the methanolic extract of *Amaranthus spinosus* linn considered as a sole source of anti-inflammatory and antioxidant. However, the isolation of active constituents from this *Amaranthus spinosus* linn species and its action responsible for its anti-inflammatory and antioxidant effect is useful for the treatment of the same in the future.

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Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 971-981
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