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RESEARCH ARTICLE

COMPARATIVE STUDY ON METHANOLIC AND CHLOROFORM EXTRACTS OF TRACHYSPERMUM AMMI SEEDS FOR ANTIARTHRITIC AND ANTIGOUT ACTIVITY.

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Abstract

Objective: The objective of the present study is to report the in vitro anti-arthritic and anti-gout activity of methanolic and chloroform extract of *Trachyspermum ammi* seeds.

Materials and Methods: Fresh *Trachyspermum ammi* seeds were collected and extracted by using methanol and chloroform solvent by successive soxhlet for the constituents. Preliminary phytochemical screening has been done. Methanolic extract was tested for its polyphenolic content by Folin-ciocalteu's reagent. In vitro anti-arthritic activity was performed by inhibition of protein denaturation method. In vitro anti-gout activity was performed by the metal chelating complex assay.

Results: The study showed the presence of cardiac glycosides, carbohydrates, phytosterols, saponins, phenolic and tannins in the methanolic extract. Alkaloids, saponins, phenolics, tannins, carbohydrates in chloroform extract. The methanolic extract showed significant anti-arthritic activity than chloroform extract, this might be due to methanolic extract was rich in polyphenols. The anti-gout activity of *Trachyspermum ammi* seeds methanolic extract was also observed to be highly significant in its activity.

Conclusion: Both the methanolic and chloroform extracts of *Trachyspermum ammi* seeds were found to have significant anti-arthritic and anti-gout activity when compared to standard drug diclofenac in a dose-dependent manner.

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Introduction:-

Arthritis is an inflammation of the joints. Arthritis is considered to be secondary to the main disease, these include Psoriasis (Psoriatic arthritis), Reactive arthritis, Ehlers-danlos syndrome, Hemochromatosis, Hepatitis, Lyme disease, Sjogren's disease, Hashimoto's Thyroiditis etc. Other frequently occurring forms of arthritis include rheumatoid arthritis, lupus, fibromyalgia, and gout¹. Inflammatory cells involved in rheumatoid arthritis include CD⁴ helper cells, β -cells, macrophages and plasma cells. Key cytokines involved in rheumatoid arthritis include tumor necrosis factor (TNF- α), interleukin-1, interleukin-6 and granulocyte macrophage-colony stimulating factor (GM-CSF)². Tests for the presence of Anti-citrullinated protein antibodies (ACPA) by anti-CCP(cyclic citrullinated peptide) test and anti-MCV assay(antibodies against mutated citrullinated vimentin)³.

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Gout is usually characterized by recurrent attacks of inflammatory arthritis a red, tender, hot and swollen joint⁴. The joint at the base of the big toe is affected in about half of cases. The underlying mechanism involves elevated levels of uric acid in the blood⁵. When the uric acid crystallizes and the crystal deposits in joints, tendons, and surrounding tissues than an attack of gout occurs⁶. Diagnosis may be confirmed by seeing the characteristic crystals in joint fluid or tophus. Treatment with NSAIDs, steroids or colchicines improves symptoms⁷. Long-standing elevated uric acid levels (hyperuricemia) may result in other symptomatology including hard, painless deposits of uric acid crystals known as tophi⁸. Lesch-Nyhan syndrome is often associated with gouty arthritis. Urate is the end product of purine metabolism. In recent years important urate transporter proteins such as the human URAT1 transporter (hURAT1) and the fructose transporter SCL2A9 have been characterized. Urate crystals lead to activation of the NALP3 inflammasome with the release of proinflammatory cytokines among them interleukins 1, 18 and 8 of the tumor necrosis factor attracting more polymorphonuclear neutrophilic granulocytes (e10-e12).⁹

Trachyspermum ammi seeds have been widely used as a traditional medicine for many diseases like cholera, diarrhea, atonic dyspepsia, indigestion, flatulence, cold, flu and other viral infections. It can be used as tonic, diuretic, expectorant, relaxes spasms and increases perspiration. Ajwain seeds contain about 50% thymol, a well known antibacterial essential oil, along with thyme can be used to enhance the immune system.

Materials and Methods:-

Collection and extraction of medicinal plant material

Fresh seeds of *Trachyspermum ammi* seeds were collected from East Godavari district (A.P) in the months of Dec-Jan 2016. The trailing herb was authenticated by Dr.J.Suneetha, Dept.of Botany, Govt Art's College, Rajahmundry, A.P., and Reference: Glossary of the presidency of Madras by Gambel, Pg.no559, Vol-1. Collected seeds were shade dried and made into the coarse powder with the use of mixer grinder. The powder was weighed and stored.

Soxhlation procedure:

Seeds were extracted with methanol and chloroform by successive soxhlation for the constituents. 200 gram of air-dried powdered material drug was successively separated in Soxhlet apparatus using methanol followed by chloroform. Each time before extraction with next solvent the powdered material was air-dried. All the extracts were concentrated by distilling the solvent and evaporating them to dryness at low temperature. They were then weighed and the percentage of different extractive values were calculated in term of air dried weight of the plant material. The extract obtained was vacuum dried by placing it in a Petri-plate on electric water bath (70°C), kept in an oven at 30°C for 2 hrs. The obtained extract was stored in a vacuum desiccator.

Estimation of Polyphenolic Contents:-

Reagents:

Methanolic extract was tested for its polyphenolic content. The colorimetric procedure described was followed. This is based on the principle that polyphenols give a blue color chromogen in alkaline medium with Folin- Ciocalteu's reagent, having absorption maxima at 720 nm.

Procedure:

The total soluble phenolic contents in seaweed were determined with Folin Ciocalteu reagent. To each of the seaweed extracts (100 µg/ml), 1 ml of Folin Ciocalteu reagent (1:10 v/v) was added and incubated at room temperature for 5 minutes. 1 ml of 7% sodium carbonate solution was added and incubated at room temperature for 90 minutes. The absorbance was measured at 720 nm using UV spectrophotometer. The same procedure was carried out for gallic acid (0.2-1 mg/ml) standard. The total phenol content of the extracts was obtained by using the standard curve. The total phenol content was expressed as gallic acid equivalent in %, w/w of the extracts¹⁰.

In-vitro anti-arthritis activity by inhibition of protein denaturation method:-

The test solution (0.5ml) consist of 0.45ml of Bovine serum albumin (5% W/V aqueous solution) and 0.05ml of test solution (250µg/ml).

Test control solution (0.5ml) consist of 0.45ml of bovine serum albumin (5% W/V aqueous solution) and 0.05ml of distilled water.

Product control (0.5ml) consist of 0.45ml of distilled water and 0.05 ml of test solution (250µg/ml).

Standard solution (0.5ml) consist of 0.45ml of Bovine serum albumin (5% w/v aqueous solution) and 0.05ml of Diclofenac sodium (250µg/ml).

All the above solutions were adjusted to pH 6.3 using 1N HCl. 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 buffer was added to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 416nm. ^[11-12] The percentage inhibition of protein denaturation can be calculated as,

Percentage inhibition = $[100 - (\text{optical density of test solution} - \text{optical density of product control}) \div (\text{optical density of test control})] \times 100$.

The control represents 100% protein denaturation. The results were compared with standard Diclofenac sodium (250µg/ml).¹¹

Anti-gout activity:

Metal chelating complex assay:-

The reaction mixture containing 1ml of different concentrations of extracts 10, 20, 40, 80, 100 and 120 µg ml⁻¹ in methanol was added to 0.1 ml of 2 mM ferrous chloride and 0.2mL of mM Ferrozine to initiate the reaction and the mixture was shaken vigorously and left to stand at room temperature for 10min. The absorbance of the solution was measured at 562nm. The positive controls were those using ascorbic acid and all tests were run in triplicate. The percentage chelating effect of Ferrozine Fe₂₊ complex formation was calculated from¹²

$$\frac{A_0 - A_1}{A_0} \times 100$$

A₀ is the absorbance of the control

A₁ is the absorbance of extract or standard

Results:-

Percentage yield of the extract:

Seeds of *Trachyspermum ammi* were selected for the present study. The crude drug was powdered and extracted with methanol to study the constituents present. The nature of extracts and their extractive values are reported in the following Table.1.

Table 1: Percentage yield of the extract

DRUGS	SOLVENT	COLOUR	% EXTRACTIVE VALUES
<i>Trachyspermum ammi</i>	Alcohol (95%)	Dark brown	11.5
	Chloroform	Brownish	6

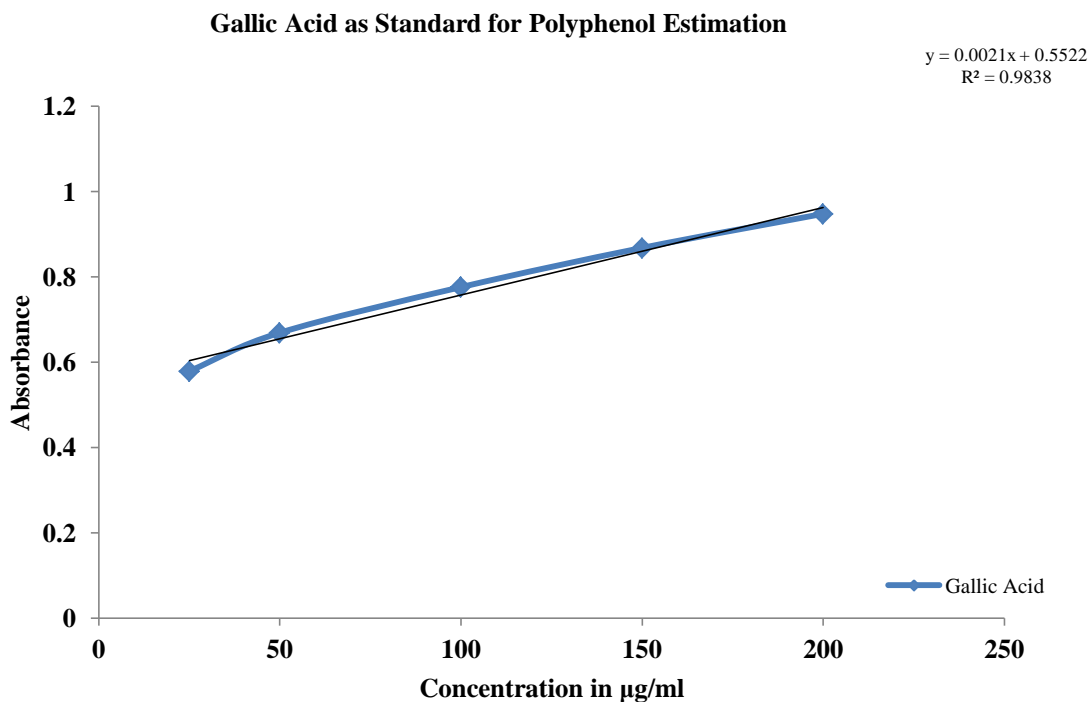
Estimation of Polyphenolic Contents:

The total phenolic content of hydro methanolic extract of *Trachyspermum ammi seed* was calculated as Gallic acid equivalent of phenols. Free radicals are produced under certain environmental conditions and during the normal cellular function in the body; these molecules are missing an electron, giving them an electric charge. To neutralize this charge, free radicals try to withdraw an electron from, or donate an electron to, a neighboring molecule. The newly created free radical in turn look out for another molecules and withdraws or donates an electron, setting off a chain reaction that can damage hundreds of molecules. The total phenol content shows good linear relation in both standard as well as sample extracts. Phenolic compounds are important plant constituents because their hydroxyl groups confer scavenging ability.

Table 2: Absorbance data of polyphenols in Gallic acid as standard

Conc. (µg/ml)	Gallic Acid
25	0.579
50	0.669
100	0.776
150	0.868
200	0.948

Fig. 1: Absorbance & concentration of Gallic acid



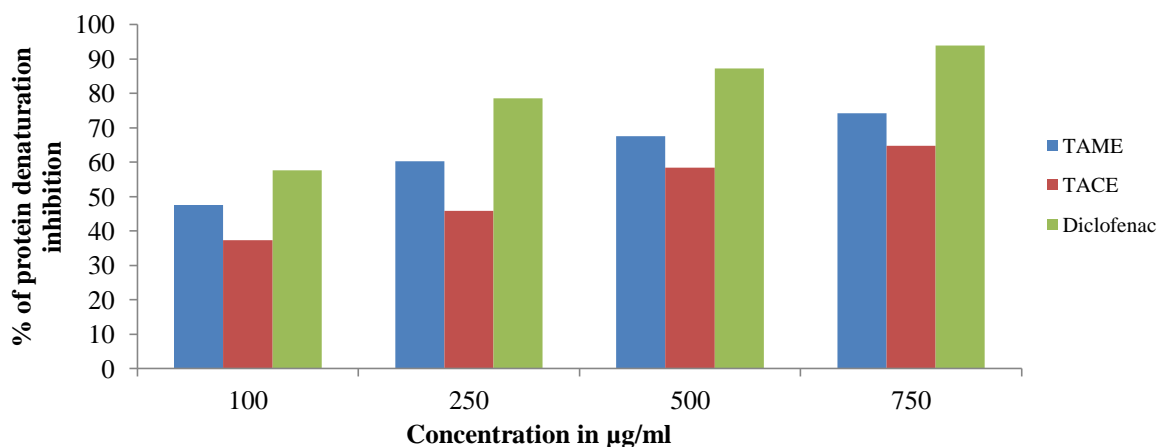
Taking into consideration from the graph, the methanolic & chloroform extracts were found to contain 63.5 µg/ml, 30.5 µg/ml of polyphenols equivalent to Gallic acid

Table 3:- Polyphenol Content of extracts equivalent to Gallic acid

S.No.	Extracts	Gallic acid Equiv. in µg/ml
1.	Methanolic Ext.	63.5
2	Chloroform Ext.	30.5

In-vitro anti-arthritic activity:

The methanolic and chloroform extracts of *Trachyspermum ammi* seeds showed good inhibitory activity on protein denaturation with the IC₅₀ value of 105.69 µg/ml for methanolic extract and 134.5 µg/ml for chloroform extract as compared to standard Diclofenac having IC₅₀ value 86.73 µg/ml. These results were shown in Table-4. Percentage inhibition of protein denaturation of methanolic & chloroform seed extracts of *Trachyspermum ammi* was shown in fig.2.

Fig. 2:- Percentage of protein denaturation inhibition of *Trachyspermum ammi* seeds extract

TAME-*Trachyspermum ammi* seeds Methanolic extract

TACE-*Trachyspermum ammi* seeds Chloroform extract

Table 4:- Anti-arthritic activity of methanolic & chloroform seed extracts of *Trachyspermum ammi*

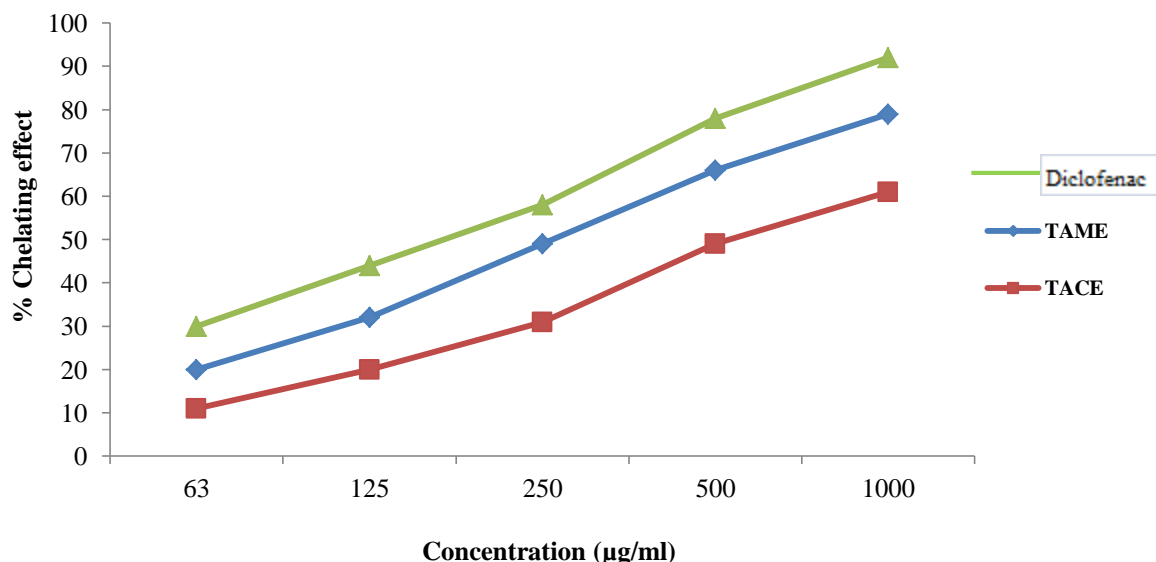
S.No	Test extract	Dose (µg/ml)	% inhibition	IC ₅₀ (µg/ml)
1	TAME	100	47.6	105.69
		250	60.3	
		500	67.5	
		750	74.2	
2	TACE	100	37.3	134.5
		250	45.8	
		500	58.4	
		750	64.7	
3	Diclofenac	100	57.6	86.73
		250	78.5	
		500	87.2	
		750	93.9	

TAME-*Trachyspermum ammi* seeds Methanolic extract

TACE-*Trachyspermum ammi* seeds Chloroform extract

Invitro anti-gout activity:

The methanolic and chloroform extracts of *Trachyspermum ammi* seed were evaluated for anti-gout activity by the metal chelating complex assay which shows the IC₅₀ value of 255.05µg/ml for methanolic extract and 510µg/ml for chloroform extract and IC₅₀ value 142 µg/ml for Diclofenac. These results were shown in Table-5. Percentage inhibition of protein denaturation of methanolic and chloroform seed extracts of *Trachyspermum ammi* seed was shown in fig.3.

Fig. 3:- Percentage Metal chelating effect of *Trachyspermum ammi* seeds**Table-5:** Anti-gout activity of methanolic & chloroform seeds extracts of *Trachyspermum ammi*

S.No	Test extract	Dose (µg/ml)	% inhibition	IC50 (µg/ml)
1	TAME	63	20	255.05
		125	32	
		250	49	
		500	66	
		1000	79	
2	TACE	63	11	510
		125	20	
		250	31	
		500	49	
		1000	61	
3	Diclofenac	63	30	142
		125	44	
		250	58	
		500	78	
		1000	92	

TAME-*Trachyspermum ammi* seeds Methanolic extract

TACE-*Trachyspermum ammi* seeds Chloroform extract

Discussion:-

Arthritis is an inflammation of the joints. It can affect one joint or multiple joints. Pathologically, rheumatoid arthritis is characterized by the infiltration of a variety of inflammatory cells into the joint. Gout is a disorder characterized by hyperuricemia and deposition of urate crystals in the synovial joints. Recent studies have clearly demonstrated the importance of medicinal plants in the treatment of many pathological diseases including arthritis & gout.

Preliminary phytochemical screening:

Seeds of *Trachyspermum ammi* were selected for the present study. The drugs were powdered and successively extracted with methanol and chloroform to study the constituents present in them. The successive extracts of the

drug were analyzed for the presence of various constituents. The result of our study on *Trachyspermum ammi* revealed the presence of amino acids in its chloroform extract and presence of alkaloids, carbohydrates, phytosterols, flavonoids and tannins in its methanolic extracts.

Determination of total polyphenols:

Both the extracts of *Trachyspermum ammi seeds* were tested for their polyphenolic content using Folin-Ciocalteu reagent using the colorimetric procedure. Many researchers have used this method to correlate the antioxidant activity of extracts to the phenolic content. This method has been used in the wine industry for over 30 years and is considered to be the best method for estimating total phenol content in complex plant products. This method is the sensitive and quantitative method, independent of the degree of polymerization compared to other methods.

The total polyphenolic content in all the extracts is expressed in terms of Gallic acid equivalent. In accordance with this report, our study indicates 63.5 µg/ml of GAE of polyphenols in the methanolic extract, whereas only 30.5 µg/ml of GAE in chloroform extracts. When compared to the results of two extracts, it reveals that the phenolic content was more with methanolic extract as compared to the chloroform extract. Using these results, levels of total polyphenols in extracts were correlated to their respective antioxidant capacity.

Screening of anti arthritic activity:

The methanolic and chloroform extracts are having an anti-arthritic potential to retard the arthritic protein denaturation, which is proving its usefulness in arthritis treatment. Percentage inhibition of protein denaturation activity of methanolic extract showed maximum inhibitory activity comparing to chloroform extract of *Trachyspermum ammi seeds*, when compared to standard Diclofenac. The Percentage inhibition of protein denaturation in terms of percentage of protection was increased in a dose-dependent manner.

Screening of anti-gout activity:

The anti-gout activity was evaluated for the metal chelating complex assay for both methanolic and chloroform extract. It was found that the percentage chelating effect is maximum for Diclofenac followed by the methanolic extract. This is evidence that the methanolic extract of *Trachyspermum ammi seed* is having anti-gout activity. The Percentage of chelating effect was increased in a dose-dependent manner.

Conclusion:-

In vitro studies on *Trachyspermum ammi seeds* extract demonstrate suppression of both arthritis and gout. Methanolic extract of *Trachyspermum ammi seeds* is a rich source of polyphenols, responsible for its significant anti-arthritic activity as compared to chloroform extract. Therefore from the present study, it can be suggested that *Trachyspermum ammi seeds* methanolic extract was proved to have anti-arthritic and anti-gout activity as compared to the standard Diclofenac, both activities are exerting in a dose-dependent manner.

The preliminary screening study showed the presence of alkaloids, carbohydrates, flavonoids, fixed oils, fats, phenolics, tannins in methanolic extract and alkaloids, saponins, carbohydrates, amino acids in chloroform extract. Hence proper isolation of the active principles might help in the findings of new lead compounds in the fields of anti-arthritic and anti-gout drug research. This study thus provided justification for the traditional usage of *Trachyspermum ammi seeds* for the treatment of arthritis, gout and may be recommended as a supplement with synthetic drugs.

Conflicts of Interest: There are no conflicts of interest.

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