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In-vitro anti-oxidant, anti-inflammatory and anti-arthritic activities in the leaves of *Coldenia procumbens* Linn

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ABSTRACT

Previous phytochemical analysis of methanolic extract of *C. procumbens L*. has indicated the presence of steroid, flavonoid and terpenoid types of compounds. Since these compounds are of pharmacological interest, coupled with the use of this plant in traditional medicine, prompted us to check *C. procumbens L* for possible antioxidant activity by DPPH, total antioxidant, total phenolic and reducing power, anti-inflammatory by HRBC membrane Stabilization method and anti- arthritic activity by the inhibition of Protein denaturation method. The percentage inhibition by DPPH method was found to be 76.26% at a concentration of 500μg/0.1ml when compared with Quercetin (87.74%). The reducing capabilities of the leaf extract of C. procumbens L was found to be in dose dependent manner which was compared with standard Quercetin. The total antioxidant activity was found to be 0.2mg equivalents of ascorbic acid. The total phenolic content was found to be 31.9mg Pyrocathecol equivalent /gm of extract. The maximum membrane stabilization of *C. procumbens L* was found to be at 98.09% and inhibition of protein denaturation was found to be at 52.84%. Therefore, our studies support the use of active constituents from *C. procumbens L* in treating inflammations and rheumatism.

Keywords: Rheumatism, Anti-oxidant, Inflammations, Denaturation, HRBC, Arthritis.

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INTRODUCTION

The uses of traditional medicine wide spread medicine and plants still represent a large source of natural anti-oxidants that might serve as leads for the development of the novel drugs. Several anti-inflammatory, digestive, antinecrotic, neuroprotective and hepatoprotective drugs have recently been shown to have an antioxidant and/ or radical scavenging mechanism as part of their activity [1]. The mechanism of inflammation injury is attributed, in part, to release of Reactive Oxygen species from activated neutrophil and macrophages. This over production leads to tissue injury by damaging the macromolecule and lipid peroxidation of membranes. In addition, ROS propagate inflammation by stimulating the release of the cytokines such as interleukine- I, tumor necrosis factor-α, and interferon-γ, which stimulate recruitment of additional neutrophil and macrophages. Thus free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation. Most clinically important medicine belongs to steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of inflammation related diseaseses. Though these have potent activity and long term administration is required for treatments of chronic diseases. Furthermore, these drugs have various and severe adverse effects. Therefore, naturally originated agents with very little side effects are desirable to substitute chemical therapeutics. Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease predominantly affecting the joints and peri-articular tissues. RA still remains a formidable disease, being capable of producing severe crippling deformities and functional disabilities. RA is classified as an inflammatory arthritis, the disease comprises of 3 basic inter-related processes like inflammation, synovial proliferation and joint tissue destruction. RA factor containing immune complexes found in thejoints activate the pathological process. Tumour necrosis factor alpha (TNF-alpha) is the product of macrophages has been demonstraded to play an important role in the pathogenesis of RA [2-3].

C. procumbens L (Family: Boraginaceae) is a flat growing herb usually lying on the ground. Stems reaching 45cm, long, shaggy; branches often numerous; young parts silky with white hairs. *C. procumbens L* is found widely in south India and its leaves are applied to rheumatic swelling. It is used in external application for causing suppuration of boils. In folklore medicine it is used to treat rheumatic swellings, in mature abscesses, leucorrhoea, menorrhagia, anti-diabetic, anti-arthritic and hypotensive [4]. Arthritis is a disease of the joints characterized by pain, Swelling, redness, heat, and at times, structural changes. Considering the indigenous uses of the plant, the present study deals with the investigation of *in vitro* anti-oxidant, anti-inflammatory and anti-arthritic activities in the leaves of the *C. procumbens L*.

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EXPERIMENTAL METHOD

Collection and extraction of medicinal plant material

The raw material of medicinal plant *C. procumbens L* was collected from different regions around Chennai and authenticated by Dr.P.Jayaraman (botanist), Director PARC, West Tambaram, Chennai. Voucher specimen [No: PARC/2008/187], deposited in our college herbarium for future reference. The dried powdered leaves of the plant materials were extracted separately with methanol using soxhlet apparatus for 48 hrs. The solvent was distilled at lower temperature under reduced pressure and concentrated on water bath to get the crude extract which is stored in desiccator for future use.

Determination of DPPH radical scavenging activity

DPPH scavenging activity was measured by the spectrophotometric method. A stock solution of 25mg of DPPH (150μM) was prepared in 100ml of ethanol. 0.1ml of extract of different concentration (31.25, 62.5, 125, 250, 500, 1000 and 2000μg/0.1ml) and 1.9ml of DPPH was added. Control without test compound was prepared in an identical manner. In case of blank, DPPH was replaced by ethanol. The reaction was allowed to be completed in the dark for about 20 minutes. Then the absorbance of test mixtures was read at 517nm.The percentage inhibition was calculated and expressed as percent scavenging of DPPH radical [5-6]. Quercetin (31.25, 62.5,125,250,500,1000 and 2000μg/0.1ml) was used as standard. The percentage DPPH inhibition was calculated from the following formula.

%DPPH inhibition= [(OD of control-OD of test) ÷ (OD of control)] X100

Determination of total antioxidant activity

The total antioxidant activity was evaluated by Prieto et al. An aliquot of 0.1ml of sample solution / ascorbic acid equivalent to 500μg was combined with 1ml of the reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). In case of blank, 0.1ml of methanol was used in place of sample. The tubes were capped and incubated in a boiling water bath at 95°C for 90 minutes. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695nm against blank [7]. The antioxidant activity was expressed as equivalents of ascorbic acid (μg/g).

Determination of total phenolic content

Total soluble phenolic content of the methanolic leaf extract was determined with folin-ciocalteu reagent using Pyrocathecol as standard. 1ml of methanolic extract (500μg) in a volumetric flask was diluted with distilled water (46ml). Folin-ciocalteu reagent (1ml) was

added and the contents of the flask were mixed thoroughly. After 3 minutes, 3ml of sodium carbonate (2%) was added then the mixture was allowed to stand for 2 hour with intermittent shaking. The absorbance was measured at 760nm [8]. The concentration of total phenolic compound in the leaf extract was determined as microgram of Pyrocathecol equivalent by using an equation that was obtained from standard curve of Pyrocathecol

Absorbance = 0.001 X Pyrocathecol (μg/ml) + 0.003

Determination of reducing power

For the measurement of reductive ability we investigated the Fe^{+3} to Fe^{+2} transformation in the presence of the samples such as leaf extract of *C. procumbens L,* Quercetin at different dose (250,500,1000,2000μg/ml) using the method of Oyaizu (1986).Definite amount of the extract and standard drugs,2.5ml phosphate buffer pH 6.6, 2.5ml 1% potassium Ferro cyanide were incubated at 50ºC for 20 minutes, 2.5ml of 10% trichloro acetic acid(TCA) were added to the mixture and centrifuged for 10 minutes at 3000 rpm. After centrifugation 2.5ml of the supernatant were diluted with 2.5ml of water and shaken with 0.5ml freshly prepared 0.1% ferric chloride. The absorbance was measured at 700nm.The control solution was prepared as above, but contained water instead of samples [9-11]. An increase in absorbance indicated higher reductive ability.

***Invitro* anti-inflammatory activity by HRBC membrane stabilization method**

The principle involved here is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. The assay mixture contains 1ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hypo saline [0.36 %], 0.5 ml HRBC suspension[10 % v/v]) with 0.5 ml of plant extracts of various concentrations (31.25, 62.5, 125, 250, 500, 1000, 2000 μg/0.5ml), standard drug diclofenac sodium (250, 500 1000, 2000 μg/0.5ml) and control [distilled water instead of hypo saline to produce 100 % hemolysis] were incubated at 37^{o} C for 30 min and centrifuged respectively[12]. The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. The percentage hemolysis produced in the presence of distilled water was taken as 100 % (table:3).The percentage of HRBC membrane stabilization or protection was calculated using the formula,

PERCENTAGE STABILIZATION = 100 – [(OPTICAL DENSITY OF DRUG) ÷ (OPTICAL DENSITY OF CONTROL) × 100]

***Invitro* anti-arthritic activity by inhibition of Protein denaturation method**

The Test solution (0.5 ml) consist of 0.45ml of Bovine serum albumin (5 % W/V aqueous solution) and 0 .05 ml of test solution (250 μg/ml). Test control solution (0.5 ml) consist of 0.45ml of bovine serum albumin (5 % W/V aqueous solution) and 0 .05 ml of distilled water. Product control (0.5 ml) consists of 0.45ml of distilled water and 0.05 ml of test solution

(250µg/ml). Standard solution (0.5 ml) consists of 0.45ml of Bovine serum albumin (5 % w/v aqueous solution) and 0.05ml 0f 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 min and the temperature was increased to keep the samples at 57° c for 3 min [13-14]. After cooling, add 2.5 ml of phosphate buffer to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 416 nm.

The percentage inhibition of protein denaturation can be calculated as,

PERCENTAGE INHIBITION = [100-(OPTICAL DENITY OF TEST SOLUTION – OPTICAL DENSITY OF PRODUCT CONTROL) ÷ (OPTICAL DENSITY OF TEST CONTROL)] ×100.

The control represents 100 % protein denaturation .The results were compared with Diclofenac sodium (250µg/ml). The percentage inhibition of protein denaturation of different concentration was given in table 4.

RESULTS

DPPH radical Scavenging Activity

Radical scavenging activity of methanolic extract of *C. procumbens L* was tested by its ability to bleach the stable DPPH radical. This method is based on the reduction of alcoholic DPPH solution in the presence of hydrogen donating anti-oxidant(AH) due to the formation of non- radical form DPPH – H by the reaction DPPH+AH→ DPPH –H +A. The remaining DPPH measured after certain time, corresponds inversely to the radical scavenging activity of the anti-oxidant. The sensitivity of the method is determined by the strong absorption of DPPH. This method is rapid, a sample analytical method which takes only 15minutes and little man power, no expensive reagents or sophisticated instruments are required [15]. This assay is being used widely as a preliminary test which provides information on the reactivity of test compound with a stable free radical since odd electron of DPPH gives strong absorption band at 517nm(violet colour) and when it is quenched by the extract , there is a decrease in absorbance. Methanolic extract of *C. procumbens L* showed a very good anti radical activity in scavenging DPPH radical (comparable to the standard, Quercetin) with a maximum inhibition of about 76.26 (IC_{50}=75µg/0.1ml) at a concentration of 500mcg/0.1ml. (Table-1, Figure-2)

Total Antioxidant Activity

The total anti-oxidant activity of methanolic extract of *C. procumbens L* was found to be 0.2mg equivalents of ascorbic acid. This may be due to the presence of alkaloids, steroids, phenolic compounds, proteins and flavonoids. From the results it is made clear that methanolic extract of *C. procumbens L* possess free radical scavenging activity through total antioxidant content [16].

Total Phenolic Content

Phenolics are very important plant constituents because of their scavenging capability due to their hydroxyl groups. It has been revealed that various phenolic antioxidants such as flavonoids, tannins, coumarins, xanthones and more recently procyanidins scavenge radicals dose dependently, thus they are viewed as promising therapeutic drugs for free radical pathologies. In the present study total phenolic compound of methanolic extract of *C. procumbens L* at 500µg was found to 31.9 µg of Pyrocathecol equivalent/gram of extract respectively [17].

Reductive Ability

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. For the estimation of the reductive ability we investigated the Fe^{3+} to Fe^{2+} transformation using the method of Oyaizu, where the change in the optical density of the final mixture is measured at 700nm (Table-2). Increase in optical density indicates higher reductive ability [18]. The reducing capabilities of the leaf extract of *C. procumbens L* was found to be in dose dependent manner when compared with Quercetin. (Figure-3)

Anti-inflammatory activity

The investigation is based on the need for newer anti-inflammatory agents from natural source with potent activity and lesser side effects as substitutes for chemical therapeutics. The percentage protection of methanolic extracts was 98.09% at 2000µg/ml (Table-3). It possesses significant activity comparable with that of the standard Diclofenac sodium. *C. procumbens L* has significant anti-inflammatory activity which may be due to presence of chemical profile such as Flavones, Tri-Terpenoids, Flavonones and Phenols [19].

Anti-arthritic activity

The methanolic extract fabricates significant activity at 52.84% at 250µg/ml by inhibition of protein denaturation and its effect was compared with the standard drug Diclofenac sodium (Table: 4 and figure: 4). The production of auto antigen in certain arthritic disease may be due to denaturation of protein. From the results of present study it can be stated that methanolic extract is capable of controlling the production of auto antigen and inhibits denaturation of protein in rheumatic disease [20].

CONCLUSION

Rheumatoid arthritis is a chronic inflammatory disease, morphological triad affecting synovium, cartilage and bone. The causative factor may be exogenous (Infections) or endogenous. *Invitro* studies on *C. procumbens L* demonstrate suppression of both inflammation and arthritis. The methanolic extracts of the leaves of *C. procumbens L* must contain some

principles, which possess anti-inflammatory and anti-arthritic activities. From the preliminary screening study, it showed the presence of Flavonones, Flavones, Tri-Terpenoids and Phenolics. Hence proper isolation of the active principles might help in the findings of new lead compounds in the fields of anti-arthritic and anti-inflammatory drug research. . Studies related to active constituents on lipid derived eicosanoids, enzyme expression (COX_2, lipoxygenase) and cytokines are necessary to understand the mechanism of action in relation to the observed anti-inflammatory activity. Hence it can be used as a potent agent against it. Further *invivo* studies are required to elucidate its exact mechanism of action.

Table1: Determination of DPPH Scavenging Activity

Concentration (µg/0.1ml)	% Inhibition	
	Coldenia procumbens	Quercetin
31.25	30.98	80.99
62.5	35.05	86.63
125	74.77	86.43
250	73.77	87.18
500	76.26	87.74
1000	67.20	88.02
2000	51.77	84.96
IC_{50}	75µg/0.1ml	

Table2: Determination of Reducing Power

Concentration (µg/0.5ml)	%Inhibition	
	Coldenia procumbens	Quercetin
250	88.82	90.52
500	72.43	92.83
1000	57.86	94.70
2000	21.82	95.95

Table 3:Anti-Inflammatory Activity of *Coldenia Procumbens*

S.NO	SUBSTANCE	DOSES (µg/0.5ML)						
		31.25	62.5	125	250	500	1000	2000
1.	Diclofenac Sodium	-	-	-	82.74%	88.39%	90.19%	99.94%
2.	*Coldenia procumbens*	86.58%	95.26%	95.48%	96.27%	96.41%	96.51%	98.09%

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Table 4: Anti-Arthritic Activity Leaves of *Coldenia procumbens*

S.NO	DRUG	PERCENTAGE INHIBITION
1.	Diclofenac Sodium(250 µg/ML)	94.22%
2.	Methanolic Extract Of *Coldenia procumbens*(250 µg/ML)	52.84%

Fig.1: Leaves of Coldenia procumbens

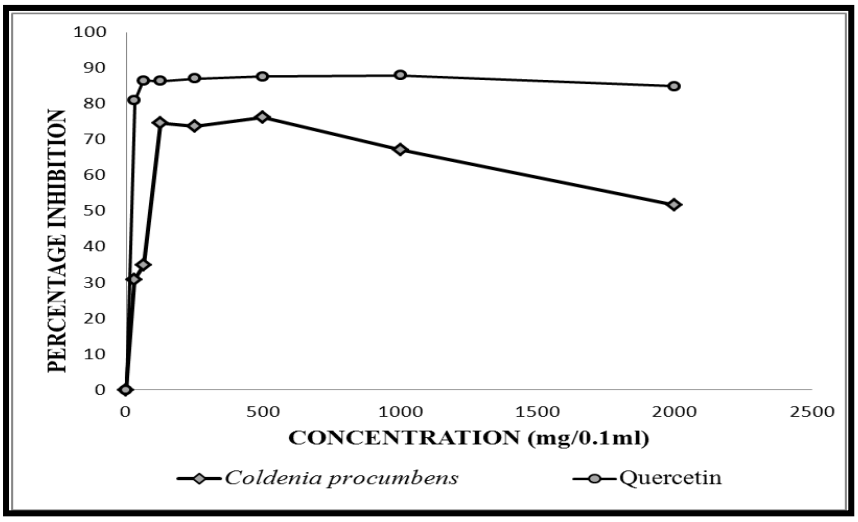

Fig.2: Anti- oxidant activity of *Coldenia procumbens*

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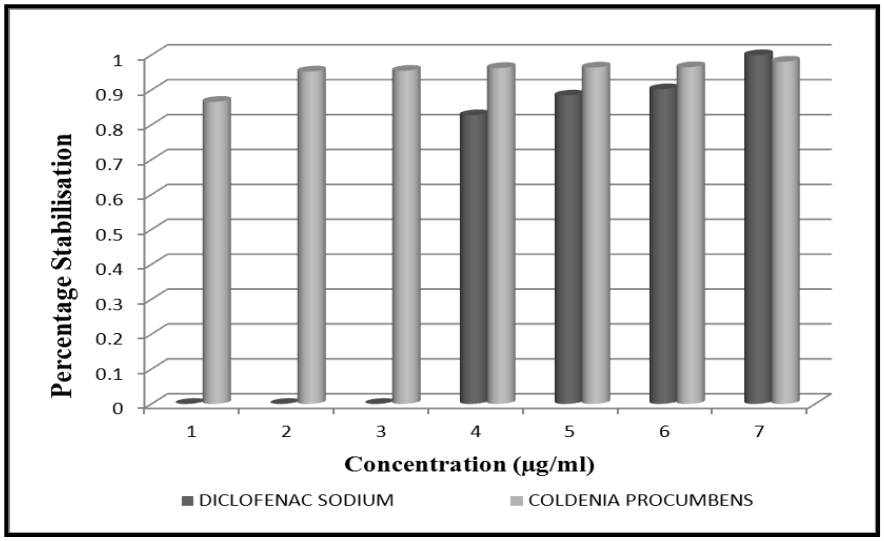

Fig.3: Anti-inflammatory activity of *Coldenia procumbens*

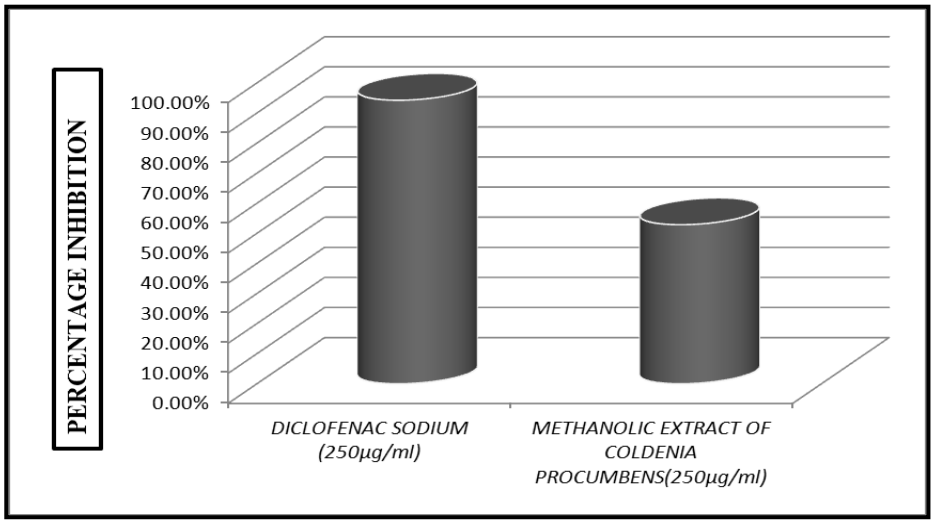

Fig.4: Anti-arthritic activity of *Coldenia procumbens*

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