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INVITRO ANTIOXIDANT ACTIVITY AND ANTI -INFLAMMATORY ACTIVITY OF ETHANOLIC LEAF EXTRACT OF COCCULUS HIRSUTUS

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ABSTRACT

Aim is to study the invitro antioxidant and anti-inflammatory action of the ethanolic extract of leaves of Cocculus hirsutus. Preliminary Phytochemical anlalysis showed the presence of flavones, flavonoids, triterpenoids, anthocyanins, coumarins, proteins, reducing sugars, alkaloids, tannins, glycosides and quinines. The invitro antioxidant study was done by DPPH, Nitric oxide radical generation and reducing power assay. The anti-inflammatory study was done by HRBC (Human Red Blood Cell) assay. Ethanolic extract of the leaf of the plant showed significant antioxidant activity and the anti inflammatory activity. The maximum percentage of inhibition by DPPH method was found to be 56.73 % at the concentration of 800 µg/ml and the maximum percentage of inhibition by Nitric oxide method was found be 58.6 %. The invitro anti inflammatoy activity was found to 79.77 % at the concentration of 25 µg and it increases with increase in concentration at the concentration of 800 µg it was found to be 94.74%. The antioxidant and the antinflammatory activity is may due to the presence of flavonoids. From the above it was concluded that the ethanolic leaf extrac of cocculus hirustus having in vitro antioxidant and anti-inflammatory activity. Further investigation has to be done in experimental animals to find out the mechanism of action.

KEY WORDS: Cocculus hirsutus, antioxidant, HRBC, inflammation.

INTRODUCTION

Among the innumerable gifts of nature belong the fascinating varieties of natural products which in some guise have been inseparable parts of mankind's history since they fulfill many of our basic requirements. Plants are an important source of natural products, thus form the

very beginning of this existence, man has familiarized himself with plants and used them in a variety of ways. Therefore some plants came to be widely used as food, while others showed beneficial effects against various human sufferings such as injuries and diseases continues at an accelerating pace and the number of new plant-derived drugs increases likewise. Globally there are wide spread diseases like diabetes, hypertension, atherosclerosis, urolithiasis, ulcers etc, which are mainly caused by increase in free radicals. Plants constitute one of the major scavenging free radicals. Hence the efficacy of scavenging property of plants will help to curb the disease. A wide range of antioxidants both natural and synthetic has been proposed for uses in treatment of human diseases. Cocculus hirsutus is a climbing scandent shrub with softly villous young parts found in India, Sudan, China and Baluchistan provinces of Pakistan. [1]

Taxonomic classification of COCCULUS HIRSUTUS (Linn) Diels [2]

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Ranunculates

Family: Menispermaceae

Genus: Cocculus

Species: hirustus

Vernacular names[3]

Eng: Broom creeper, ink berry

Hin: Patalagarudi, Jaljamini

Kan: Sogadibali, Quesaribali

Mar: Patalagaruda kkoti

San: Patalagaredah, chilihindah

Tam: Kattukkoti

Tel: Dusaratiga

Morphology

Cocculus hirsutus Diels (locally known as Jamti-ki-bel) belongs to family Menispermaceae. It is a climbing shrub. The leaves of this plant are sometimes sublanceolate, retuse or obtuse, and mucronate; sometimes three-lobed, base subcordate or truncate, with young villosus on both surfaces; the petiole is half inch long. The roots are very crooked and twisted upon

themselves, keeled, seldom branched with a peculiar, acrid odour and a disagreeable and bitter taste.[4]

The species of Cocculus is used as ingredients in various formulations in Ayurveda system of medicine as "Guduchi Satwam" It heads the list of valuable bitter tonics in Ayurvedic pharmacopoeia and is the bitterest amongst them. It is recommended for various ailments like rheumatism, urinary disease, diabetes, dyspepsia, splenic affections, chronic gonorrhoea, diarrhea, dysentery and vermifuge. Traditionally the root and leaves of Cocculus hirustus have more medicinal value. The juice of the leaves coagulates in water and it is used as cooling agent in eye problem and soothing agent in eczema, Impetigo and dyspepsia. Decoction of root used in rheumatism.[5]

Recently many studies investigated on the Antidiabetic,[6],[7] anti- inflammatory,[8] analgesic, diueretic,[9], [10] antimicrobial,[12],[13] immunostimulant,[14]spermatogenic[15],anticancer[16] activities. From the literature review it is concluded that the ethanolic extract of leaves of Cocculus hirustus (Linn) Diels is not investigated for invitro antioxidant and anti inflammatory effect.

MATERIALS AND METHODS

Plant collection

The plant cocculus hirsutus was collected from Tirunelveli and authenticated by Botany Professor Dr. V.Chelladurai.

Preparation Of Extract: The Leaves were separated, shade dried and powdered in a grinder. The powder was extract with ethanol using soxhlet apparatus. The residue was macerated overnight with water and filtered. The filterate was dried on tray drier at 60^0 C and was used for study purpose.

Phyto_Chemical Screening[17]

The ethanolic extract of leaves of Cocculus hirustus was studied for various phyto chemical analysis.

Test for Steroids

1 mg of test extract was dissolved in few drops of chloroform. 3ml of acetic anhydride was added followed by a drop of conc.sulphuric acid. Bluish-green colour showed presence of steroids.

Test for Flavones

To the extract in alcohol, a few magnesium turnings and a few drops of conc. HC1 was added. Boiled for 5 minutes. Red coloration showed presence of flavones.

Test for Phenolic Compounds

To the extract few drops of alcoholic ferric chloride solution was added. Bluish-green colour indicates presence of phenolic compounds.

Test for Triterpenoids

Extract was warmed with tin and thionyl chloride. Pink colour indicates presence of triterpenoids.

Test for Flavones

To the extract, 10% Sodium hydroxides was added. Yellow to orange colour showed presence of Flavanones.

Test for Anthocyanins:

To the extract 10 % Sodium hydroxide was added. Blue colour showed presence of anthocyanins.

Test for Reducing Sugars

Extract was mixed with Fehiling's solution I & II. Red coloration indicated the presence of reducing sugars.

Test for Anthraquinone Glycosides:

Extract was macerated with ether and filtered, to the filtrate caustic soda was added. Pink, violet or red colour in aqueous layer indicates the presence of anthraquinones. If present as a glycoside then the test should be modified by hydrolysing with hydrochloric acid as first step.

Test for Alkaloids

Few drops of acetic acid was added to little of extract, then followed by Draggendroff's regent and shaken well. Orange red precipitate indicates the presence of alkaloids.

Test for Quinones

To the little amount of extract sodium hydroxide was added, red or blue green colour indicates the presence of Quinones.

Test for Tannins

To the extract, little amount of lead acetate solution was added. White precipitate indicates presence of tannins.

Test for Saponins

A little of the extract was shaken with water. Copious lather formation confirmed presence of saponins.

Test for Amino acids

To the extract added Ninhydrin reagent and warmed it. Violet or Pink colour indicates the presence of amino acids.

Invitro Antioxidant Assay

DPPH radical scavenging activity [18]

1 ml each concentration (25-800 µg/ml) of test sample solution was added 1.9 ml DPPH in ethanol solution. After vortexing, the mixture (due to quenching of DPPH free radicals) was measured at 517 nm and the percentage inhibition was calculated. The IC_{50} values were determined as the concentration of the test mixture that gave 50% reduction in the absorbance from a control blank. The experiment was repeated in triplicates and the results were expressed in mean. The results were computed for analysis.

Effect of Cocculus hirsutus on inhibition of nitric oxide radical generation [19]

Aqueous sodium nitro prusside at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrate, which can be estimated by use of Greiss reagent. Sodium nitro prusside (5 mM) in phosphate buffered saline was mixed with 3 ml of different concentrations (50-1000 µg/ml) of the extracts dissolved in methanol and incubated at 25^{o}C for 150 min. The samples from the above were allowed to react with Greiss reagent (1% sulphanlimide, 2% H_3PO_4 and 0.1% napthylethylenedrocholoride). The absorbance of the chromophore formed during the diazotization of nitric with sulphanilamide and subsequent coupling with napthylethylenediaminehydrochloride was read at 546 nm. The experimental were replaced in triplicates. The percentage scavenging of nitric oxide radical activity was calculated by the formula below and results were computed.

$$\% \text{ Nitic oxide scavenged} = \frac{(\text{OD of control} - \text{OD of test})}{\text{OD of control}} \times 100$$

Reducing Power Assay [20]

The reducing power of ethanolic extract of leaves of **Cocculus hirsutus** was determined by using various concentrations of the extracts in 1 ml of deionised water mixed respectively with phosphate buffer (2.5ml, 0.2M, pH 6.6) and 1% potassium ferricyanide (2.5ml). The mixture was incubated at 50^{o}C for 20 minutes. Aliquots of TCA (2.5ml, 10%) were added to the mixture. Which was then centrifuged at 1036 × g for 10 minutes. The upper layer of reaction mixture was mixed with distilled water (2.5ml) and freshly prepared ferric chloride solution (0.5ml, 0.1%). The absorbance was measured at 640nm. Increased absorbance of the reaction mixture indicated reducing power. Reducing power is given in terms of ascorbic acid equivalent (AsEmg-1). The experiment was carried in triplicate.

In Vitro Anti – Inflammatory Activity – HRBC Membrane Stabilization Method [21]

Stabilization of Human Red Blood Cell Membrane method is based on the principle of hypotonicity induced membrane lysis. The lysosomal enzymes released during inflammatory condition produce a variety of disorders. The extracellular activity of these enzymes in said to be related to acute or chronic inflammation. The anti inflammatory agents acts by either inhibiting the lysosomal enzymes or by stabilizing the lysosomal membranes. Since the human blood cell membrane are similar to lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis is taken as a measure of anti – inflammatory activity of drug. Diclofenac 100µg/ml concentration was used as standard drug.

The assay mixture contains 1 ml of phosphate buffer (P_H 7.4), 2 ml hyposaline, (0.36%), 0.5 ml HRBC suspension (10% v/v) and 0.5 ml of the ethanolic extract of the plant in various concentrations (25, 50, 100, 200, 400 and 800) and standard drug Diclofenac and control (distilled water instead of hypo saline to produce 100% hemolysis) were incubated at 37^{o}C for 30 minutes, and centrifuged. The hemoglobin content in the supernatant solution was estimated using spectrophotometer at 560nm. The percentage hemolysis produced in the presence of distilled water was taken as 100%. The percentage of HRBC membrane stabilization or protein was calculated using the formula:

$$\% \text{ protein} = 100 - \frac{\text{optical density of drug treated sample}}{\text{Optical density of control}} \times 100$$

RESULT

1. Preliminary phytochemical screening

Phytochemical screening reveals that ethanolic extract showed the presence of flavones, flavonoids, triterpenoids, anthocyanins, coumarins, proteins, reducing sugars, alkaloids, tannins, glycosides and quinines. (Table – I)

Table-I Preliminary Phytochemical Screening Of cocculus hirsutus

+: Present, - : Absent

Phytochemicals	Ethanolic Leaf Extract
STEROIDS	-
TRITERPENES	+
FLAVANOIDS	+
FLAVONES	+
ANTHOCYANINS	+
COUMARINS	+
PROTEINS	+
REDUCING SUGARS	+
ALKALOIDS	+
TANNINS	+
PHENOLICS	-
GLYCOSIDES	+
QUINONES	+
SAPONINS	+
ANTHRAQUINONES	+

2. In Vitro Antioxidant Activity

DPPH Free Radical Scavenging Activity

The scavenging activity of the ethanolic extract of leaves of Cocculus hirsutus and ascorbic acid on DPPH radical was studied. This method is based on the reduction of alcoholic DPPH solution in the presence of antioxidant(AH).

DPPH +AH ⟶ DPPH-H + A

The remaining DPPH measures indirectly the antioxidant capacity of the extract. The reducing capacity of the plant extract was found to increasing with dose dependent manner when compared with ascorbic acid. The plant extract showed significant scavenging effect and it increase with increase in concentraion but the activity was less when compared to standard ascorbic acid. IC 50 value of ethanolic leaf extract was found to be 551.74 µg/ml, given in Table –II

Table -II – In vitro antioxidant - DPPH ASSAY

Concentration	Percentage Of Inhibiton	
	Cocclus Hirustus	Ascorbic Acid
25	6.59	80
50	21.34	86
100	29.08	88
200	30.04	90
400	49.14	92
800	56.73	94
IC 50	551.74 (µg/ml)	

Nitric Oxide Scavenging Activity of Cocculus hirsutus

Nitric oxide is a free radical produced in mammalion cells, involved in the regulation of various physiological process; however excess production of nitric oxide is associated with several diseases. In the present study the nitrate produced by the incubation of solution of sodium nitropursside in a standard phosphate buffer at 25^{o}C was reduced by ethanolic extract. This may be due to the antioxidant activity. It was observed that nitrate free radical were scavenged by the extract in a concentration dependent manner (Table –III). The maximum percentage inhibition in nitricoxide method was found to be 58.6% at the concentration of 800 µg/ml the antioxidant activity was due to the presence of tannis, flavonoids, and glycosides.

Table III – Invitro antioxidant- Nitric oxide radical generation method

S.No	Concentration	Percentage Of Inhibiton	
		Cocclus irustus	Ascorbic Acid
1.	25	11.89	25.13
2.	50	22.64	34.17
3.	100	28.73	44.93
4.	200	36.65	55.46
5.	400	43.06	66.72
6.	800	58.6	75.25
	IC 50	582.53 (µg/ml)	

Invitro anti -inflammatory activity[22] [23]

Ethanolic extract of leaves of Cocculus hirsutus were subjected to in – vitro anti inflammatory activity in various doses, they showed dose dependent anti-inflammatory activity. At 100 µg/ml concentration HRBC membrane stabilization was found to 86.32 % and reaches maximum at the concentration of 800 µg/ml by 94.74%. The effect may be due to presence of flavonoids and terpenoids.

Table IV – In vitro anti inflammatory activity of Cocculus hirustus– HRBC assay

Concentraion	Percentage Of Hrbc Protein	
	Cocclus Hirustus	Diclofenac
25	79.77	
50	82.39	
100	86.32	80.25
200	90.60	88.89
400	92.51	93.65
800	94.74	98.32

Cocculus hirsutus (L) Diels, a twig with leaves and flowers.

DISCUSSION

The ethanolic leaf extract of the plant Cocculus hirustus showed significant antioxidant and antiinflammatory effect, this was mainly due to the presence of flavonoid and tri terpenoids. Proper isolation of the active principles can help in the findings of new lead compound that having anti-inflammatory action. Further investigations are needed to prove antinflammatory action with experimental animals

CONCLUSION

From the research it was concluded that the ethanolic leaf extract of the Cocculus hirustus leaves having invitro antioxidant and anti-inflammatory activity.

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