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In-vitro protease and hyaluronidase inhibitory activities of *Crotalaria pallida* seed extracts

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Abstract
In the present study, three solvent extracts (ethyl acetate, acetone and methanol) of *Crotalaria pallida* seeds were prepared and evaluated for antioxidant, protease inhibition and hyaluronidase inhibitory activities. All the three extracts showed significant inhibition among the tested activities. In DPPH assay, methanol extract has showed highest radical scavenging activity with IC_{50} value of 114 µg/mL in comparison to acetone and ethyl acetate extracts. In TAA, acetone extract has showed highest antioxidant activity with 17 µg/mL (measured in terms of GAE). In FRAP assay, methanol extract has showed significant reduction potential with 113 µg/mL. Further, in hyaluronidase inhibition assay, acetone extract has showed highest inhibition with IC_{50} value of 443 µg/mL and in protease inhibition assay, methanol extract has showed maximum inhibition with IC_{50} value of 459 µg/mL.

Keywords: *C. pallida*, anti-oxidant activity, protease inhibition, hyaluronidse inhibition, anti-inflammation

Introduction
Oxidative stress is induced by increase in the levels of ROS levels as a result of chemical substances exposure or other environmental stress. When the intrinsic antioxidant system within an organism is damaged, it is difficult to remove free radicals which lead to oxidative stress and this result in various chronic diseases.[1]. A state of chronic oxidative stress leads to damage of various cellular components, cellular membranes, DNA and proteins. It is also results in the chronic inflammatory responses through a number of various intracellular signaling pathways, which finally results in a variety of pathological conditions including cardiovascular diseases, autoimmune disorders, and aging.[2]. Excessive ROS generation triggers the activation of underlying signaling mechanisms which results in the activation of nuclear transcription factor κB (NF-κB), which acts as a transcriptional regulator of the innate immune system and stimulate the release of a variety of pro-inflammatory cytokines from various tissues.[3,4].
Eventhough inflammation is a self-defence mechanism exhibited by organisms to defend against various external stimuli, prolonged inflammation can lead to development of other serious pathological conditions. The inflammatory response is marked by the stimulation of macrophages and succeeded by the increase in the secreations of nitric oxide (NO), pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), and cell adhesion molecules [5]. These ROS mediated inflammatory responses can be encountered by antioxidants present in the natural sources. Recent research reports also indicated that plant based antioxidants are of great importance as therapeutic agents to reduce oxidative stress associated chronic diseases including inflammatory diseases. [6].
Hyaluronidase enzyme catalyses the hydrolysis of hyaluronic acid which is distributed extensively in extracellular matrix of soft connective tissues including skin, umbilical cord and synovial fluid [7]. The hyaluronidase enzyme plays an important role in inducing inflammation [8]. In-*vivo* activation of hyaluronidase enzyme by metal ions such as calcium results in the degranulation of mast cells and it leads to the release of inflammatory mediators. Hyaluronic acid acts as a viscous lubricating agent which is present in the synovial fluid of joints. Excessive degradation of hyaluronic acid by hyaluronidase results in the depletion of hyaluronic acid is observed in rheumatoid arthritis. An imbalance between the synthesis of hyaluronic acid and its degradation by hyaluronidase leads to joint disease and other types of inflammatory conditions.[9]. Thus inhibition of hyaluronidase is an important target for maintaining balance in hyaluronic acid metabolism. Therefore, hyaluronidase inhibitors may play a major role in inhibiting the inflammatory process.
Proteolylis is a crucial mechanism in certain physiological processes, and it is strictly regulated. Numerous studies have shown that protease play a key role in homeostasis, fertilization, antigen presentation and apoptosis. Furthermore, they are essential for regulation of immunological mechanisms such as immune and inflammatory cell migration and activation and antigen presentation [10].Various studies have showed the role of protease in human diseases such as cancer, thrombotic and inflammatory disorders. This implies that proteases are responsible for the pathogenesis of various diseases, thus they are good targets for curing such diseases with protease inhibitors.[11] Protease inhibitors can be found in all living beings from microorganisms to multicellular organisms and play a important role by preventing excessive or unnecessary proteolytic activities that are injurious to cells or organs or organism [12].
Serine proteases are involved in different physiological processes like immune responses, blood clotting, apoptosis and tissue healing and also extensively involved in immunological responses and pro-inflammatory actions. Serine proteases act as modulators of the immune system by regulating cytokine and chemokine production. T cells and NK cells produce IL-32, which is a pro-inflammatory cytokine. Natural compounds such as phytoestrogens,

flavonoids, and protease inhibitors have shown to provide defence against inflammatory diseases through the regulation of inflammatory pathways. Soybean Bowman-Birk trypsin inhibitor (BBI) is a small protein with 71 amino acids and various reports also showed that BBI has an anti-inflammatory activity against most of the diseases including ulcerative colitis, encephalomyelitis, multiple sclerosis, and the autoimmune neuritis. [13].

Trypsin is one of the serine proteases plays a key role in the normal physiological functions of the cells, e.g. protein maturation, digestion, blood coagulation, control of blood pressure, immune response and apoptosis. The cure of such diseases by protease inhibitors from natural sources, therefore, provides an attractive target for pharmaceutical research [14].

Medicinal plants provide a rich source of new anti-inflammatory drugs and antioxidants which also play an important role in treating many chronic diseases has been extensively studied from past few decades. Because synthetic drugs have side effects, natural compounds having lesser side effects from plants origin have gained an importance. The plant *Crotalaria pallida* belongs to the family Fabaceae is an annual erect herb approximately 1.50 m in height and it mainly grows extensively in tropical and subtropical regions of India [15]. The stems yield fibre similar to sunn hemp, and the seeds are used as a substitute for coffee [16]. The plant is used as a good cover crop in tea, coconut and rubber plantations to prevent soil erosion and also used as a green manure [17]. Different parts of this plant are used in folk medicine to treat urinary infections and the swelling of joints. [18].

Materials and Methods

Chemicals

Hyaluronidase, 2, 4, 6-Tripyridyl-S-triazine (TPTZ), 2, 2-diphenyl-1- picrylhydrazyl (DPPH), Ascorbic acid were procured from Sigma Aldrich (St. Louis, MO, USA). Trypsin was purchased from Himedia, India. All other reagents used in this study were of analytical grade.

Collection of seeds and preparation of solvent extract

C. pallida seeds were collected in Tumakuru (13.3379º N, 77.1173º E), Karnataka State, India. The seeds were dried and powdered. Solvent extracts were prepared by extracting the powdered seeds using different solvents separately. The seeds powder (20 gm) was extracted using soxhlet apparatus with ethyl acetate, acetone and methanol solvents separately. The extract was filtered and concentrated under vacuum at 40°C. The extracts were stored at 4°C until further use.

Phytochemical screening

Tannins

Three drops of 5% ferric chloride was added to 1mL of extract. Greenish black precipitate indicates the presence of tannins.

Phenolics

The phenolic content of the extract was determined by the method of Singleton *et al.* [19]. The extract was mixed with 1 mL of 5% sodium carbonate. To this mixture, 0.25 mL of Folin–Ciocalteau reagent (1:1 diluted with water) was added, mixed thoroughly and incubated for 60 min at room temperature. After incubation, absorbance was measured at 725 nm using spectrophotometer. The increase in optical density of the test samples when compared to the blank was taken as an indication of the presence of phenolics. Tannic acid, a known phenolic, was taken as a standard.

Saponins

The extract (1mL) was taken in a test tube was vigorously shaken for 2 min. Both the persistent frothing for 5 min and the frothing observed after heating on water bath indicated the presence of saponins.

Steroids

The presence of steroids was determined by Liebermann's Burchard test. The extract (1mL) was dissolved in 0.5mL of acetic anhydride and cooled in ice. This was mixed with 0.5 mL of chloroform, and then 1mL of concentrated H_2SO_4 was carefully added using a pipette, along the sides of the test tube. At the junction of two liquids, the appearance of a reddish brown ring indicates the presence of steroids.

Terpenoids

One mL of the mixture containing 0.5 g of 2,4-dinitrophenylhydrazine in 100 mL of 2 M HCl was added to 1mL of extract. Yellow–orange colouration indicated the presence of ketonic terpenoids.

Flavonoids

The presence of flavonoids was determined by Shibata's reaction method. The extract (1mL) was warmed with three pieces of magnesium turnings and mixed with three drops of concentrated HCl. Orange–pink colouration indicated the presence of flavonoids [19].

In vitro antioxidant activities

Total antioxidant activity

Total antioxidant activity was done according to the method of Prieto *et al.* [20]. Different concentrations (0 - 250 µg) of *C. pallida* seed extracts were mixed with 1 mL reagent solution (0.6 M H_2SO_4, 28mM sodium phosphate and 4 mM ammonium molybdate in the ratio of 1:1:1) and the mixture kept in boiling water bath at 95° C for 60 min. After cooled, the absorbance was read at 695 nm. Total antioxidant activity was expressed as gallic acid equivalents (GAE).

FRAP (ferric reducing antioxidant power) assay

The FRAP assay of *C. pallida* seed extracts was determined according to the method of Benzie and strain [21]. FRAP reagent was prepared by the addition of 300 mM acetate buffer (10 mL), TPTZ dissolved in 40 mM HCL and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1. Different concentrations (0-500 µg) of *C. pallida* seed extracts were incubated with 1 ml of reagent for 30 min at 37 ˚C. The absorbance was read at 593 nm. FRAP was expressed as GAE.

Diphenyl-1-Picrylhydrazyl- (DPPH) assay

Radical scavenging ability of the *C. pallida* seed extracts was performed according to the method of Braca *et al.* [22]. Different concentrations (0-250 µg) of *C. pallida* seed extracts were incubated with DPPH (1 mM) solution for 30 min at 37°C. After incubation, the absorbance was read at 517 nm against blank sample of DPPH alone. Ascorbic acid was used as positive control. The percent of radical scavenging activity was calculated using the following formula

$$RSA(\%) = \frac{Abs\ control - Abs\ sample}{Abs\ control}$$

Protease inhibition

Protease inhibitory assay was carried out according to Satake *et.al.* [23] by using casein as the substrate (2% in 200 mM Tris-HCl buffer; P^H 7.4). Briefly different concentrations (0 -500 µg) of *C. pallida* seed extracts were pre-incubated with trypsin at 37 ℃ for 30 minutes. Then the reaction mixture was incubated with the substrate for 2 hours at 37℃. The reaction was terminated by adding trichloroacetic acid (TCA) and the mixture was allowed to stand for 30 minutes. The mixture was then centrifuged for 3000 rpm for 5 min. One mL of the supernatant was mixed with 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of 1:2 diluted FC reagent and further incubated for 20 minutes at room temperature. The decrease in the intensity of color developed in the reaction mixture was measured at 660 nm against blank. The percent protease inhibitory activity was calculated using the following formula

$$\%\ inhibition = \frac{Abs\ control - Abs\ sample}{Abs\ control}$$

Hyaluronidase inhibition

Hyaluronidase inhibition study was done according to the method of Reissig *et.al.* [24]. Different concentrations of extracts (0-500 µg) were pre-incubated with 25 µL (37.5 units) of hyaluronidase (1500 IU/mL) in total volume of 300 µL reaction mixture containing 0.2 M sodium acetate buffer (pH 5.8) with 0.3 M NaCl and incubated for 30 min at 37°C. After pre-incubation, hyaluronic acid substrate was added to initiate the reaction. The reaction mixture was incubated for 2 hours at 37°C. After incubation, 20 µL of 0.8 M potassium tetraborate (pH 9.1) was added to stop the reaction. The mixture was kept in boiling water bath for 3 min and cooled to room temperature. Later 1.5 mL of p-DMAB was added and incubated for 20 min at 37°C. Absorbance was monitored at 585 nm.

$$\%\ inhibition = \frac{Abs\ control - Abs\ sample}{Abs\ control}$$

Results and discussion

Phytochemical screening

Medicinal plants provide an excellent source of phytoconstituents having potential antioxidant activity, protease and hyaluronidase inhibitory activities. Phytochemical molecules from the medicinal plant extract can be a rich source for isolation of active molecule for drug discovery [25]**.** Phytochemical analysis of the all the three solvent extracts of *C. pallida* seeds revealed the presence of different phytoconstituents. Methanol and acetone extracts showed the presence of phenolics, flavanoids, tannins, terpenoids and glycosides. Ethyl acetate extract showed the presence of phenolics, tannins and glycosides. Results are tabulated in table 1.

In-vitro antioxidant activities

Total antioxidant activity

Solvent extracts of *C. pallida* seeds (methanol, acetone and ethylacetate) were evaluated for antioxidant activities including DPPH, TAA, and FRAP, in order to understand the possible mechanisms responsible for antioxidant activities. In TAA, Mo (VI) will be reduced to Mo (V) by the antioxidants present in the sample and it leads to the formation of green colored complex at low pH. Among the three extracts tested, acetone extract has showed highest TAA of 17±0.1 µg/mL (expressed as GAE µg/mL of extract) followed by methanol extract with 13±0.4 µg/ mL and ethyl acetate with 10±0.2 µg/mL.TAA is increased gradually with the increasing concentration of extracts from 0-500 µg/ml. The results are tabulated in table 2.

FRAP

In the FRAP assay, antioxidants present in the seed extracts will donate the hydrogen atom there by breaking the free radical chain. Thus it acts as an important indicator of reduction potential [26]. The results confirm potent reduction potential of all the three extracts of *C. pallida* seeds. Among them, methanol extract has showed highest reducing potential of 113±0.6 µg/ mL, which was gradually increased with the increasing concentration measured in terms of GAE µg/mL extract. Acetone extract has showed 32±0.4 µg/mL followed by ethyl acetate extract with 17±0.4 µg/mL. The results are tabulated in table 2.

DPPH assay

DPPH is a stable free radical and it is based on its reduction by the antioxidant is a commonly used method for studying the antioxidant capacity of plant seed extracts. All the three extracts of *C. pallida* seed showed potentially high tendency to scavenge the DPPH as showed in fig. 1 in a dose dependent manner. Methanol extract has showed highest radical scavenging activity among the three extracts and its percentage of inhibition reached up to 91.87 (IC_{50} value 114 µg/mL) followed by acetone extract with 49.45 % (IC_{50} 266.53 µg/mL). Ethyl acetate extract has showed minimum inhibition of 36.92 with (IC_{50} 303.33 µg/mL). Similar to our results, many authors have attributed the antioxidant potential of plants to higher DPPH radical scavenging activity. [26]

Protease inhibition

Inhibitors of serine proteases play an important role in antagonizing inflammation. Several trypsin inhibitors from different sources such as plants, microorganisms, marine organisms have been isolated and their structures been elucidated. The inhibitory effect of plant polyphenols on the activity of trypsin has also been reported in the literature [27]. Among the three solvent extracts of *C. pallida* seeds, again methanol has shown highest protease inhibition of 81.11% with IC_{50} value of 459 µg/mL followed by acetone extract with IC_{50} value of 545.81 µg/mL. Among the tested extracts the ethyl acetate extract did not showed significant inhibition even at concentration of 500 µg.

Hyaluronidase inhibition

Hyaluronidase enzyme is considered as a mediator of most of the pathological and physiological conditions including inflammation. Hyaluronidase inhibitors play an important role in anti-inflammatory activity. Hyaluronic acid (HA) is a component of the extracellular matrix (ECM) that exists as a high-molecular-weight polymer. Depending on its size, hyaluronic acid has various structural and biological functions. High molecular weight hyaluronic acid is

responsible for lubrication, tissue hydration and free radical sequestration while smaller fragments generated by hyaluronidase act as signaling molecules which activates the inflammatory response by up-regulating the various cytokines, CD44 receptors and matrix metalloproteinases. Thus balance between hyaluronic acid and its degradation is required for the normal functioning of the connective tissue and inhibition of hyaluronidase could aid in the restoration of tissue homeostasis.

Discovery of hyaluronidase inhibitors could be valuable for developing anti-tumour agents.[28] In addition to this, hyalyronidase inhibitors are responsible for release of histamines from mast cells, because of this they are also useful in regulating type–I allergy.[29]. Plants with anti-hyaluronidase have been previously reported *Aesculus hippocastanum, Hedera helix Anemarrhena asphodeloides*, *Rubus fruticosus* have exhibited anti-hyaluronidase activity. Among the documented hyaluronidase inhibitor, plant derived bioactive components are of great importance. Wide research on hyaluronidase inhibition showed that antioxidants such as phenolic acids and flavanoids possess significant anti-hyaluronidase activities [9].

The extracts showed inhibition in a dose dependent manner. Among the three extracts tested, acetone extract has showed highest inhibition of up to 61.96% with IC_{50} value of 405.11 µg/ml followed by methanol extract with 56.8% with IC_{50} value of 443 µg/ml and then ethyl acetate with 38.50% with IC_{50} value of 697 µg/ml. The hyaluronidase inhibitors play prominent role in regulating metabolism of hyaluronic acid. Most of the hyaluronidase inhibitors have been identified, among them, plant derived bioactive principles are of much importance. Phenolic acids and flavanoids which are the well-known antioxidants possess strong hyaluronidase inhibition. [23].

Conclusion

From all these experiments, it is evident that, all the three extracts of *C. pallida* seeds are rich sources of hyaluronidase inhibitory bioactive compounds due to its ability to inhibit hyaluronidase enzyme. These extracts are also good sources of hyaluronidse inhibitors and as well as anti-oxidants. The bioactivities, exhibited by these plant extracts reflect the use of extracts as active agents in treating inflammation related dieseases. Methanol and acetone extracts of *C. pallida* seeds also exhibited significant inhibition against proteases which could be used as therapeutic targets in some disease conditions like cancer, thrombotic and inflammatory disorders.

Figures and Tables

Table 1: Phytochemical analysis of *C. pallida* seed extracts.

Phytochemicals	Ethyl acetate	Acetone	Methanol
Phenolics	+	+	+
Saponins	-	-	-
Flavanoids	-	+	+
Tannins	+	+	+
Terpenoids	-	+	+
Glycosides	+	+	+

Note: +, present; -, absent;

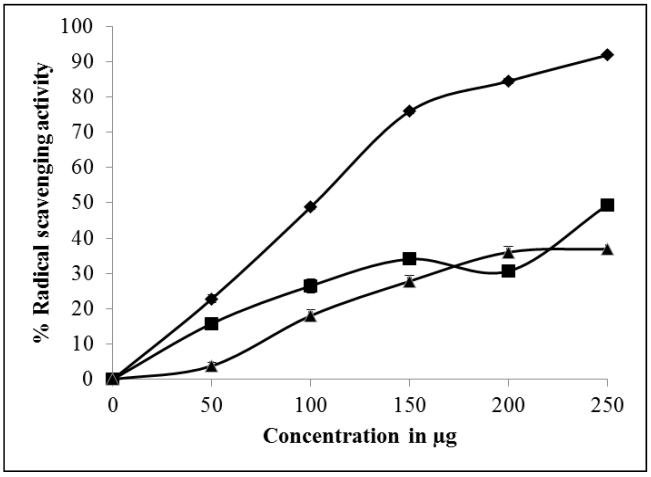

Fig 1: DPPH radical scavenging activities of different extracts of *C. pallida* seed.

DPPH was incubated with different concentrations of *C. pallida* seed extracts (methanol-■, acetone-♦, Ethylacetate-▲) at 37 ˚C for 30 min. The absorbance was read at 540 nm. Each value is the mean ± SD of three independent experiments.

Table 2: IC_{50} value for *C. pallida* seed extracts for DPPH radical scavenging activity.

C. pallida seed extracts	IC_{50} value (µg/ml)
Methanol	114
Acetone	266.53
Ethyl acetate	303.33

The IC_{50} value was calculated for *C. pallida* seed extracts (methanol, acetone and ethylacetate) for radical scavenging activity using DPPH method. The IC_{50} was expressed as µg/ml.

Table 3: Total Antioxidant activities (TAA) and FRAP of *C. pallida* seeds extracts.

Concentration of extract (µg/ml)	TAA of *C. pallida* seed extracts (GAE) (mean ± SD)			FRAP of *C. pallida* seed extracts (GAE) (mean ± SD)		
	Methanol	Acetone	Ethylacetate	Methanol	Acetone	Ethylacetate
100	4±0.2	9±0.2	3±0.4	51±0.8	7±0.4	5±0.4
200	6±0.2	12±0.2	5±0.4	79±1.6	17±0.6	8±0.4
300	9±0.2	13±0.1	7±0.4	101 ±2.3	24±0.8	10±0.6
400	12±1.3	15±0.4	8±0.2	108±1.4	28±0.6	14±0.4
500	13±0.4	17±0.1	10±0.2	113±0.6	32±0.4	17±0.4

GAE: Gallic acid equivalents µg/ml of extract. Each value represents mean ± SD of three independent experiements.

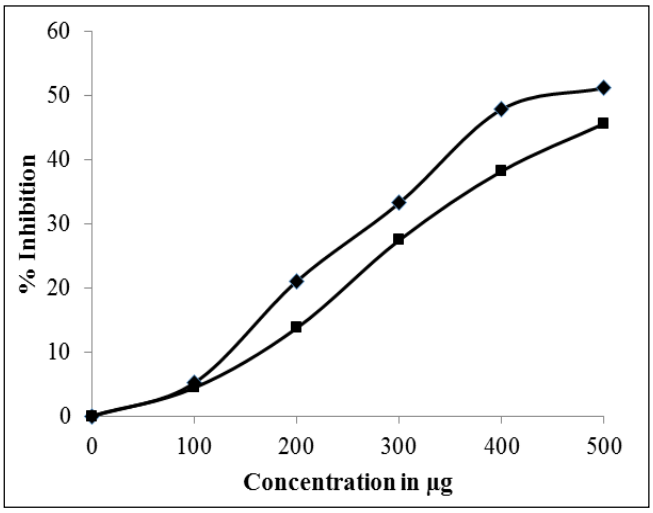

Fig 1: Protease inhibition of *C. pallida* seed extracts

Trypsin was pre-incubated with different concentrations of (methanol-♦, acetone-■) *C. pallida* seed extracts at 37 ˚C for 30 min. Later casein was treated with the reaction mixture and incubated at 37°C for 2 hrs 30 min. Absorbance was monitored spectrophotometrically at 660 nm. Values are mean ± SD of triplicate determinations.

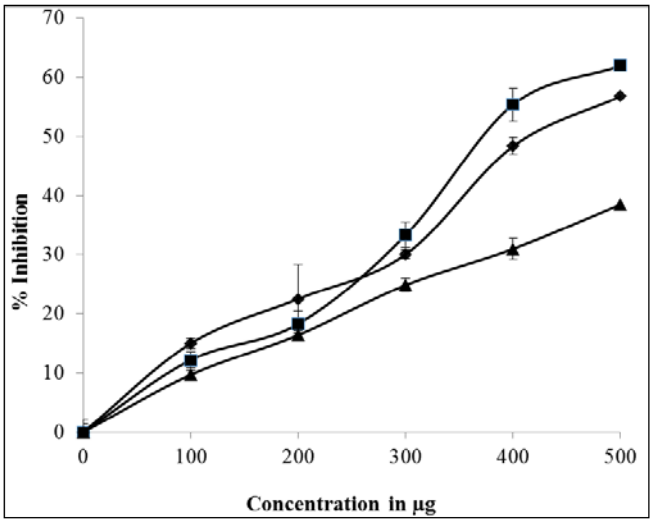

Fig 2: Hyaluronidase inhibition by *C. pallida* seed extracts.

Hyaluronidase (37.5 units) was preincubated with varied concentrations of methanol [♦], acetone [■] and ethyl acetate [▲] extracts at 37°C for 30 min. The reaction was initiated by adding substrate hyaluronic acid (30µg) in sodium acetate buffer (pH 5.8) and incubated at 37°C for 2 hrs. Absorbance was monitored spectrophotometrically at 580 nm. Values are mean ± SD of triplicate determinations.

Table 3: IC_{50} value of *C. pallida* seed extract for hyaluronidase inhibition study.

***C. pallida* seed extracts**	**IC_{50} value (µg/ml)**
Methanol	443
Acetone	405.11
Ethyl acetate	697

The IC_{50} value was calculated for ***C. pallida*** seed extracts (methanol, acetone and ethyl acetate) for hyaluronidase inhibition. The IC_{50} was expressed as µg/ml.

Table 3: IC_{50} value of *C. pallida* seed extract for protease inhibition study.

***C. pallida* seed extracts**	**IC_{50} value (µg/ml)**
Methanol	459
Acetone	545.81

The IC_{50} value was calculated for ***C. pallida*** seed extracts (methanol and acetone) for protease inhibition. The IC_{50} was expressed as µg/ml.

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