

Comparative study on anti-oxidant and anti-inflammatory activities of *Caesalpinia crista* and *Centella asiatica* leaf extracts

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Abstract

Background:

Amyloidosis, oxidative stress and inflammation have been strongly implicated in neurodegenerative disorders like Alzheimer's disease. Traditionally, *Caesalpinia crista* and *Centella asiatica* leaf extracts are used to treat brain related diseases in India. *C. crista* is used as a mental relaxant drink as well as to treat inflammatory diseases, whereas *C. asiatica* is reported to be used to enhance memory and to treat dementia.

Objective:

The present study is aimed to understand the anti-oxidant and anti-inflammatory potential of *C. asiatica* and *C. crista* leaf extracts.

Materials and Methods:

Phenolic acid composition of the aqueous extracts of *C. crista* and *C. asiatica* were separated on a reverse phase C18 column (4.6 x 250 mm) using HPLC system. Antioxidant properties of the leaf extracts were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and the reducing potential assay. The anti-inflammatory activities of aqueous extracts of *C. crista* and *C. asiatica* were studied using 5-lipoxygenase assay. Polymorphonuclear leukocytes (PMNLs) were isolated from blood by Ficoll-Histopaque density gradient followed by hypotonic lysis of erythrocytes.

Results:

Gallic, protocatechuic, gentisic, chlorogenic, caffeic, p-coumaric and ferulic acids were the phenolic acids identified in *C. crista* and *C. asiatica* leaf aqueous extracts. However, gallic acid and ferulic acid contents were much higher in *C. crista* compared to *C. asiatica*. Leaf extracts of *C. asiatica* and *C. crista* exhibited antioxidant properties and inhibited 5-lipoxygenase (anti-inflammatory) in a dose dependent manner. However, leaf extracts of *C. crista* had better antioxidant and anti-inflammatory activity compared to that of *C. asiatica*. The better activity of *C. crista* is attributed to high gallic acid and ferulic acid compared to *C. asiatica*.

Conclusions:

Thus, the leaf extract of *C. crista* can be a potential therapeutic role for Alzheimer's disease.

KEY WORDS: Anti-inflammation, antioxidant, *Caesalpinia crista* and *Centella asiatica* leaf extract, lipoxygenase, reversed phase-high performance liquid chromatography

The oxidative stress and inflammation have been implicated in neurodegenerative disorders like Alzheimer's disease (AD) and Parkinson's disease, among others.[1,2] The generation of reactive oxygen species, which are toxic, is a part of normal metabolism in a biological system. The balance between the production of reactive oxygen species and anti-oxidants is essential in a biological system to prevent adverse effects of oxidative stress. The imbalance between reactive oxygen species and antioxidants in the human body leads to oxidative stress.[3] The oxidative stress has detrimental effects on central nervous system.[2] The brain is more prone to oxidative stress because of (i) brain is rich in easily oxidizable unsaturated fatty acids; (ii) brain requires more oxygen per unit weight (20% of total oxygen requirement in human beings); (iii) brain is also rich in iron and ascorbate which are key players in oxidation; and (iv) brain is deficient in antioxidants. The free radicals thus generated are known to attack macromolecules such as deoxyribonucleic acid, proteins, lipids and carbohydrates. This leads to either onset or acceleration of degenerative disorders.[4] Overall, the oxidative modification of biomolecules leads to the imbalance in the metabolic activities in AD.[5] The accumulating evidences on the role of oxidative stress suggest that it may be an early event in the onset of AD.[6] The defense system of the human body against oxidative stress induced impairments includes antioxidant enzymes and non-enzymatic antioxidant proteins.[7] These inbuilt defense system are reduced in AD.[8] The inflammation is another responsible factor in AD and is presumed to be mediated through the cross talk among the amyloid, astrocytes and microglia.[9] These reactions lead to altered neuronal function and the inflammatory injury. The leukotrienes and prostaglandins are the mediators of inflammatory response in the cell.[10] 5-lipoxygenase enzyme is important in the biosynthesis of leukotrienes and its location is confined to polymorphonuclear leukocytes (PMNLs), monocytes, macrophages, mast cells and B-lymphocytes.[11] Since 5-lipoxygenase mediates a key step in the generation of inflammatory molecules, modulation of its activity has therapeutic implications.[12] For screening the compounds for anti-inflammatory actions, 5-lipoxygenase provides a good *in vitro* model.

There are reports on use of plant extracts as antioxidants in animal models to test their efficacy.[13] *Caesalpinia crista* Linn (Syn. *C. bonducella* L. Roxb.) belongs to family Fabaceae, found abundantly in tropical and subtropical regions of Southeast Asia. The kernel of *C. crista* contains cassane- and norcassane-type diterpenoids.[14] The stem and roots are also known to contain new type of diterpenes.[15] The tribal knowledge base in India insighted that aqueous extract of *C. crista* is used as stress relaxation health drink by forest dwellers. There are limited studies on anti-diuretic, antibacterial, anti-diabetic and anti-oxidant potential properties of *C. crista*.[16] The dried leaves of Indian penny wort (botanical name: *Centella asiatica*) is mixed with milk and consumed to improve memory[17] and this is practiced traditionally in selected regions in India.[18] There are studies on diverse effects of *C. asiatica* such as acetylcholine-esterase inhibition, antioxidant, neuroprotection and amyloid load reduction.[19,20,21]

We have reported for the first time that aqueous extracts of *C. crista* not only inhibited the amyloid fibril formation, but also could dis-aggregate the pre-formed fibrils, while *C. asiatica* partially inhibited the amyloid fibril formation.[20,22] The current trend in controlling AD is multi-target directed ligands approach. The single molecule can possess multiple activities such as anti-amyloidogenic, anti-oxidant and anti-inflammatory activities. Since we have already reported the anti-amyloidogenic potential of *C. crista* and *C. asiatica*[20,22] the present study is undertaken to study the effects of the aqueous extracts of both *C. crista* and *C. asiatica* leaves for their antioxidant and anti-inflammatory activities.

Materials and Methods

Chemicals and plant material

1,1-Diphenyl-2-picrylhydrazyl (DPPH), adenosine triphosphate (ATP), dithiothreitol (DTT), arachidonic acid (AA), nordihydroguaiaretic acid (NDGA) from Sigma Chemical Co., MO, USA. All other chemicals and solvents used are of analytical grade.

Preparation of aqueous extracts of *C. asiatica* and *C. crista*

C. crista was obtained from Western Ghats of Karnataka, India and the species of the plant was identified by the Botanist (taxonomic deposit number: 417358). The *C. asiatica* was procured from local vegetable market, Mysore and it was identified by authenticated botanist (Taxonomic deposit number is 9831. The aqueous extract of leaves was prepared. A total of 40 g of dried leaf was washed in triple distilled water. The washed leaf was boiled in steam extractor (2 L of triple distilled water for ~ 3 h until the aqueous content become half). The extract was filtered through Whatman No. 42 filter paper to get the clear solution and lyophilized to powder (yield – 2.5%, w/w).[20,22]

Determination of total phenolics in aqueous extract of *C. asiatica* and *C. crista*

Total phenolics in aqueous extract of *C. asiatica* and *C. crista* were determined according to the method followed by Swain and Hillis.[23] Gallic acid was used as a standard. The total polyphenols content in the extract was expressed as gallic acid equivalents (GAE).

Identification of phenolic compounds in aqueous extract of *C. asiatica* and *C. crista* by high performance liquid chromatography

Polyphenols in the aqueous extracts of *C. asiatica* and *C. crista* were separated on a reverse phase C18 column (4.6 mm × 250 mm) using HPLC system (Agilent-Model 1200 series) using diode array detector (operating at 280 nm and 320 nm). A solvent system consisting of water: methanol: acetic acid (83:15:2) was used as mobile phase (isocratic) at a flow rate of 1 ml/min.[24] Known quantities of phenolic acid standards such as caffeic acid, p-coumaric acid, cinnamic acid, ferulic acid, gallic acid, gentisic acid, protocatechuic acid, syringic acid, vanillic acid were used for identification and quantification of phenolic acids present in the extract.

Antioxidant assays

DPPH radical scavenging assay

DPPH is a stable free radical that accepts an electron or hydrogen atom to become a stable 1,1-diphenyl-2-picrylhydrazine molecules. The reduction of DPPH radical was determined by a decrease in the absorbance at 517 nm. The antioxidant activity of leaf aqueous extracts of *C. crista* and *C. asiatica* and standard synthetic antioxidant ascorbic acid was measured in terms of hydrogen donating or radical scavenging ability. Briefly, 1 mL of 200 µM methanolic solution of DPPH was incubated with different concentrations of *C. crista* and *C. asiatica* extracts and standard ascorbic acid for 20 min at room

temperature. At the end of the incubation period, the absorbance was measured using an ultra violet-visible spectrophotometer at 517 nm. The percentage of scavenging or quenching of DPPH radicals (Q) by *C. crista*, *C. asiatica* and ascorbic were calculated using the following formula.

$$Q = 100 (A_o - A_c) / A_o$$

Where A_o is the absorbance of the control tube and A_c was the absorbance of the tube with 'c' concentration of the sample. All the experiments were performed in triplicates.

Reducing potential: Potassium ferricyanide reducing method

The reductive potential of the *C. crista* and *C. asiatica* extracts was determined according to the method of Oyaizu[25] Different concentrations of *C. crista* and *C. asiatica* extracts in 0.5 mL of water were mixed with equal volumes of 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide [K₃Fe (CN) 6]. The mixture was incubated for 20 min at 50°C. At the end of incubation, an equal volume of 10% trichloroacetic acid was added to the mixture and centrifuged at 3200 g for 10 min. The supernatant was mixed with distilled water and 0.1% ferric chloride at 1:1:0.2 (v/v/v) and the absorbance were measured at 700 nm. An increase in the absorbance of the reaction mixture indicates the potential reducing power of the sample. Ascorbic acid was used as a standard for comparison.

Anti-inflammatory activity: 5-lipoxygenase assay

Isolation of 5-lipoxygenase from human PMNLs

Human peripheral venous blood from healthy persons who have not under any medical prescription was collected in a tube containing ethylenediaminetetraacetic acid. The experimental protocol adopted was approved by the Central Food Technological Research Institute's animal ethical committee. PMNLs were isolated from blood by Ficoll-Histopaque density gradient followed by hypotonic lysis of erythrocytes.[26] All the procedures were performed at 4°C. PMNLs were re-suspended in phosphate buffer saline and sonicated for 20-30 s at 20 kHz to release the cytosolic 5-lipoxygenase enzyme into solution. This solution was centrifuged at 100,000 g for 30 min at 4°C and the supernatant was used as a source of enzyme. The purity and viability of PMNLs were checked by trypan blue and Wright's straining methods, respectively. The purity and viability of PMNLs were found to be >90% and >95%, respectively. The Protein content in human PMNLs was estimated according to Lowry's method.[27]

5-lipoxygenase enzyme assay

5-lipoxygenase enzyme assay was performed using previously reported method.[28] The enzyme reaction mixture contains 100 mM pH 7.4, 50 μM DTT, 200 μM ATP, 300 μM CaCl₂, 150 μM AA and 5 μg enzyme. The aqueous leaf extracts of *C. crista* and *C. asiatica* were incubated with the enzyme for 2 min prior to the addition of AA. The enzymatic reactions were carried out at room temperature. 5-lipoxygenase activity was measured as 5-hydroxyeicosatetraenoic acid formed at 234 nm using spectrophotometer (Shimadzu).

Results and Discussion

Polyphenol content in *C. crista* and *C. asiatica* aqueous extract and identification of phenolic acids

Polyphenols are the major group of compounds that contribute to the antioxidant and anti-inflammatory properties. The total phenolic content in the aqueous leaf extracts of *C. crista* and *C. asiatica* was found to be 119.7 ± 3.5 and 73.8 ± 2.8 mg GAE/g, respectively. The polyphenols in the aqueous leaf extracts of *C. crista* and *C. asiatica* were separated on reverse phase C18 column on HPLC. Gallic acid, protocatechuic

acid, gentisic acid, chlorogenic, caffeic, p-coumaric and ferulic acids were the phenolic acids identified in *C. crista* and *C. asiatica* aqueous extracts. Gallic acid content (7391 µg/g) was more followed by ferulic acid (1694 µg/g) and p-coumaric acid (633 µg/g) in *C. crista*, whereas in *C. asiatica*, caffeic acid content (467 µg/g) was more followed by p-coumaric acid (376 µg/g) and gentisic acid (154 µg/g). We have analyzed aqueous extracts of *C. crista* and *C. asiatica* for phenolic acid composition by HPLC. The results showed that gallic acid and ferulic acid contents were much higher in *C. crista* compared with *C. asiatica*, while caffeic acid was present in both the extracts, almost in similar quantities. It should be noted that significantly high contents of gallic acid, ferulic acid and p-coumaric acid were present in *C. crista* leaf extract compared with that of *C. asiatica* extract. Gallic acid is a potential antioxidant, prevents memory deficits, neuroprotective agent against oxidative stress,[29,30,31] whereas ferulic acid is a potential antioxidant, it exerts an antidepressant-like effect and it protects brain tissues against cerebral ischemic injury.[32,33,34] On the other hand, p-coumaric acid also is an antioxidant compound and it attenuates apoptosis, inhibits adipogenesis and effective antibacterial compound.[35,36,37]

Anti-oxidant assays

DPPH radical scavenging assay

To study the antioxidant properties of the leaf extracts, the DPPH scavenging potential of *C. crista* and *C. asiatica* leaf extracts were determined. We have used ascorbic acid as a positive control, due to its well-known antioxidant properties. Aqueous leaf extracts of *C. crista* and *C. asiatica* scavenged DPPH radicals in a dose dependent manner. Between the *C. crista* and *C. asiatica* leaf extracts, *C. crista* leaf extract had lower IC₅₀ value (24.35 µg/mL) compared to *C. asiatica* (139.5 µg/mL) indicating that *C. crista* had better DPPH radical scavenging activity [Table 1].

The DPPH scavenging assay is used to evaluate the free radical scavenging potential of various plant extracts.[38,39] Our results are in agreement with other studies.[40,41] The effect of antioxidants on DPPH may be due to either hydrogen donating property or electron transfer by polyphenols.[42] The DPPH radical scavenging ability of the aqueous extracts may be attributed to the polyphenols present in the extracts of *C. crista* and *C. asiatica*.

Reducing potential of *C. crista* and *C. asiatica* leaf extracts

The reducing potentials of the extracts indicate their ability to act as antioxidant. The reducing power of aqueous extracts of *C. crista* and *C. asiatica* leaves were assessed. The ascorbic acid was used as a standard. A dose dependent increase in reducing power of *C. crista* and *C. asiatica* were observed. *C. crista* extract was found to be more efficient in reducing ferric to ferrous form of iron compared to that of *C. asiatica* [Figure 1]. However, the standard ascorbic acid showed relatively higher reducing power compared to *C. crista* and *C. asiatica* [Figure 1a and b]. The iron reducing potential of a compound indicates its ability to act as antioxidant. In potassium ferricyanide reducing assay, the reductants in the extracts may reduce ferric to ferrous by donating electron. The amount of ferrous can be monitored by measuring the complex formation with Perl's Prussian blue at 700 nm.[43] Our results showed that *C. crista* has more reducing ability than *C. asiatica*. The reducing potential of extracts of *C. crista* and *C. asiatica* may be due to the electron donating property of the water-soluble polyphenols present in them. [44] Differences in their phenolic acids may be responsible for their differences in antioxidant properties.

Anti-inflammatory assay: 5-lipoxygenase assay

The anti-inflammatory activities of aqueous extracts of *C. crista* and *C. asiatica* were studied by inhibiting 5-lipoxygenase that was isolated from PMNLs assay. The results showed that *C. crista* showed significant inhibition of 5-Lipoxygenase with IC₅₀ of 23 ± 1.1 µg/mL compared to *C. asiatica* with IC₅₀ of 250 ± 2.81 µg/mL. The standard, NDGA had the lowest IC₅₀ value of 8.6 ± 0.52 µg/mL [Table 2]. These results

indicated that *C. crista* was effective as anti-inflammatory molecule compared to *C. asiatica*. The 5-lipoxygenase enzymes is a key enzyme in the biosynthesis of leukotriens. 5-lipoxygenase contains non-heme iron in the catalytic site. It catalyzes incorporation of dioxygen into unsaturated fatty acid. It mainly converts AA to biologically active leukotriens. These leukotrienes are implicated in inflammatory and allergic reactions. The harmful effects can be prevented by inhibiting its production. Hence, it can be done by inhibiting the 5-lipoxygenase, which catalyses its production. Few studies have been focused on spices and its effect on 5-lipoxygenase and leukotriene synthesis in the human PMNLs. These studies also shows that kind of inhibition by these natural molecules are reversible in nature and there lies the importance of these natural compounds in fighting against the inflammation without any side-effects, but these studies fail to explain the mechanism of action. Our results demonstrated that *C. crista* extract had better potential to inhibit 5-lipoxygenase activity compared to that *C. asiatica*. The IC₅₀ value of *C. crista* extract was closer to NDGA (standard) compared with that of *C. asiatica* extract [Table 3]. These effects may be attributed to the polyphenols present in the extracts. Our results are in agreement with other studies where phenols/flavonoid compounds in vegetable/fruits are shown to modulate activities of 5-lipoxygenase and prostaglandin-H synthase pathways of AA.[45]

Conclusion

More emphasis on search for novel natural molecules that have multi-target directed ligands such as anti-amyloidogenic, antioxidant, acetylcholine esterase inhibitors, anti-inflammatory molecules etc., is necessary to combat AD. Earlier, we have reported that aqueous extracts of *C. crista* not only inhibited the amyloid fibril formation but also could dis-aggregate the pre-formed fibrils while *C. asiatica* partially inhibited the amyloid fibril formation. In the present study, we have found that *C. crista* leaf extract exhibited better antioxidant and anti-inflammatory activities compared with that of *C. asiatica*, which may be due to the differences in their polyphenol composition and other bioactive compounds. As aqueous extract of *C. crista* has shown anti-amyloidogenic, antioxidant and anti-inflammatory activities, *C. crista* has all the potential to possibly become the drug candidate for AD.

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Footnotes

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Conflict of Interest: None declared.

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Figures and Tables

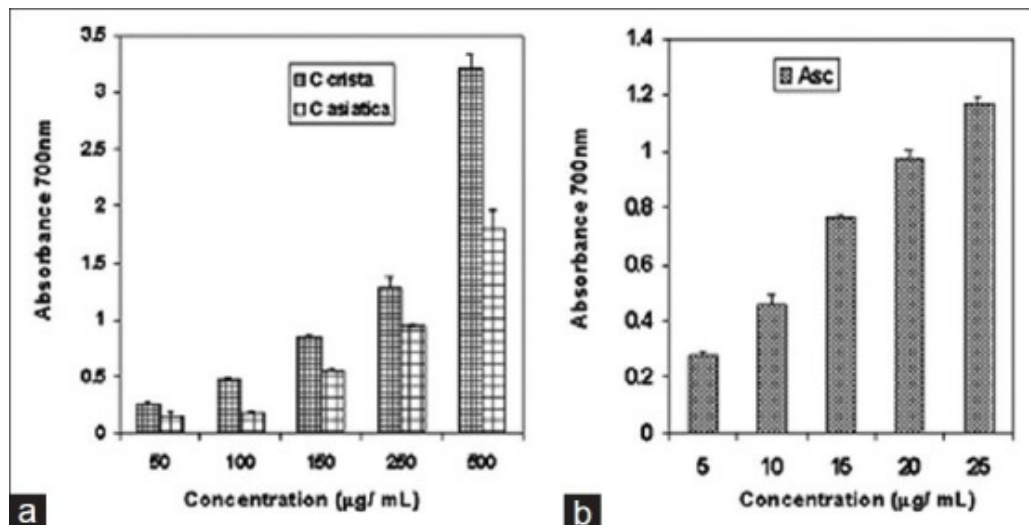
Table 1

Phenolic content in aqueous extracts of *C. asiatica* and *C. crista* as measured by HPLC

Sample	<i>C. asiatica</i> ($\mu\text{g/g}$ dry extract)	<i>C. crista</i> ($\mu\text{g/g}$ dry extract)
Gallic acid	51.13 \pm 1.53	7391.3 \pm 32.56
Protocatechuic acid	113.33 \pm 6.12	283.43 \pm 9.29
Gentisic acid	154.59 \pm 6.31	83.53 \pm 4.97
Chlorogenic acid	98.06 \pm 5.79	396.18 \pm 8.04
Caffeic acid	467.17 \pm 7.04	412.96 \pm 8.47
P-Coumaric acid	376.64 \pm 5.33	633.33 \pm 7.17
Ferulic acid	15.67 \pm 1.67	1694.42 \pm 10.99

HPLC: High-performance liquid chromatography, *C. asiatica*: *Centella asiatica*, *C. crista*: *Caesalpinia crista*

Figure 1



Reducing potential of (a) aqueous extracts of *Caesalpinia crista* and *Centella asiatica* and (b) ascorbic acid (standard)

Table 2

DPPH scavenging assay for aqueous extracts of *C. crista* and *C. asiatica**

Extract/standard	IC ₅₀ (µg/mL)
<i>C. crista</i>	24.35±1.1
<i>C. asiatica</i>	139.5±2.01
Ascorbic acid	14.75±0.72

*The inhibition by these two extracts was compared with standard ascorbic acid. *C. asiatica*: *Centella asiatica*, *C. crista*: *Caesalpinia crista*, DPPH: 1,1-Diphenyl-2-picrylhydrazyl

Table 3Inhibition of 5-lipoxygenase activity by *C. crista* and *C. asiatica* extract

Extract/standard	IC ₅₀ (µg/mL)
<i>C. crista</i>	23 ± 1.1
<i>C. asiatica</i>	250 ± 2.8
NDGA	8.6 ± 0.52

*The IC₅₀ of two extracts was compared with standard NDGA. *C. asiatica*: *Centella asiatica*, *C. crista*: *Caesalpinia crista*, NDGA: Nordihydroguaiaretic acid