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Comparative phytochemical and anti-oxidant (*in vitro*) assessment of different aerial parts *viz.* leaves, bark and pods of *Cassia fistula*

Ishita Gupta and Punita Sharma

Abstract
Cassia fistula, also known as Indian Laburnum, is found throughout Asia including India. It is a medicinal plant of great importance because of its power of healing countless disorders. The work presented in this paper is performed keeping in mind the high therapeutic value of *C. fistula.* For achieving this objective, methanol extract of different parts *viz.* bark, pods and leaves of *C. fistula* were prepared and subjected to antioxidant assay employing *in-vitro* hydrogen peroxide scavenging assay and reducing power assay. Methanol extract of the pods showing maximum anti-oxidant activity was used for isolation of bioactive compounds namely Rhein, Emodin and Chrysophanic acid (anthraquinones), Catechin (tannin) and Luteolin (flavonoid).

Keywords: Phytoconstituents, H2O2 scavenging, reduction potential, anti-oxidant

Introduction
Majority of the population of developing countries depends on their traditional medicinal system, especially plant-based, as they are less toxic and has no/less side effects [1]. *Cassia fistula* is among 3000 medicinal plants that have been used in Unani, Ayurvedic and traditional herbal medicinal system of India for curing various disorders/ailments because of its high therapeutic potential [2]. *Cassia fistula* (Amaltas) belongs to the Leguminosae family, grows throughout India and is also known as golden shower because of its beautiful bunch of yellow flowers. A moderate sized tree that grows up to 24m, ascending up to an altitude of 1,220-1300m in the sub Himalayan tract and outer Himalayas. The fruits of *C. fistula* are long cylindrical pods filled with dark brown sweet, sticky pulp consisting of seeds. *C. fistula* tree has rough and dark brown bark and its bright green leaves are pinnate. It is found in Bangladesh, China, Hong Kong, Sri Lanka, Burma, Philippines, Malaysia, Indonesia, Thailand, South Africa, Mexico, East Africa and Brazil. *C. fistula* has been reported to treat many skin, liver and gastro-intestinal disorders. It is also used to cure tumors of abdomen, glands, throat cancer, convulsions, dysuria, epilepsy, tumours, leprosy and syphilis and treats rheumatism [3-4]. Previous studies show that the plant contains anthraquinones and its derivatives, tannins, flavonoids and sterols possessing different biological activities isolated from different parts of *C. fistula* [5-9]. Rhein is the major constituent isolated from different parts of this plant and is reported to have anti-cancer activity [10]. Flavonoids and chromones exhibiting anti-tobacco mosaic virus activity (anti-TMV) were reported from the bark and stems of *C. fistula* [11-13]. Essential oil from the flowers, leaves, pulp and seeds possesses hemolytic and anti-fungal activity [12-15]. Amentoflavone, a biflavonoid, present in the leaf extract of *Cassia fistula* possesses cytotoxicity and anti-oxidant potential [16]. The plant has also been reported to possess anti-diabetic, anthelmintic, anti-tussive, anti-pyretic, anti-oxidant, hepatoprotective, anti-microbial, anti-fertility, anti-leishmmaniatic, neuroprotective, anti-ulcer activities, anti-aging properties and nephroprotective effect [17-23]. Plant extracts, now-a-days, are finding applications in various fields of research like nanoparticle synthesis, polymer synthesis etc. Synthesis of Silver nanoparticles from *C. fistula* leaf extract has been reported, which shows anti-microbial, anti-oxidant and anti-cancer properties besides their application in chemical sensors [24-25].
On the basis of phytochemical screening done previously, it was found that the pods, bark, leaves and seeds collected from the Baddi region of Himachal Pradesh are rich in phenolic compounds like Tannins, Terpenoids, Flavonoids, Steroids and Anthraquinones. However, Saponins are completely absent in the plant [26]. It was concluded that the pods of *C. fistula* is enriched with phenolic components, which is our major area of concern. So, present study aims at exploring bark, leaves and pods of *C. fistula* phytochemically and biologically.

This paper presents the preliminary anti-oxidant assessment of crude extracts of different parts of *Cassia fistula viz.* bark, leaves and pods and isolation of the phenolic constituents responsible for the anti-oxidant potential of the plant employing *in-vitro* methods like reducing power assay and hydrogen peroxide scavenging assays.

Material and Methods

All the chemicals and organic solvents used were of LR/AR grade purchased from SD Fine-Chemicals. Ceric ammonium sulphate and iodine (SD Fine) were used as visualizing spray reagents. Silica Gel (60-120; 100-200 mesh, SD Fine) used for column chromatography; precoated TLC aluminium sheets (Merck) were used for qualitative and quantitative TLC. Melting points were determined in soft glass capillaries in an electrothermal melting point apparatus. Double beam UV-VIS Spectrophotometer (LABTRONICS model LT-2700) ranging between 190-1100 nm was used. The ^{1}H-NMR spectra was recorded on Bruker Avance- II, 400 MHz NMR instrument. The chemical shifts (δ) were expressed as parts per million (ppm) and the coupling constants (J) were recorded as hertz (Hz). DMSO, $CDCl_3$ and Methanol were used as solvent and TMS was used as internal standard.

Collection, Processing and Extraction of Plant Material

Aerial parts of *Cassia fistula viz.* bark, pods and leaves were collected from the campus of Maharaja Agrasen University, Baddi, India. Plant material was shade dried and kept free from unwanted matter and contaminants. The shade dried plant material was crushed into coarse powder and was extracted with methanol in soxhlet for forty-eight hours and dried under reduced pressure to yield hot methanol extract. The dried methanol extract of the pods of *Cassia fistula* was taken for isolation of pure compounds using various chromatographic techniques like column chromatography, TLC and preparative TLC.

Isolation of Pure Compounds

For the isolation of phytoconstituents, the crude methanol extract of the pods of *Cassia fistula* was subjected to repeated column chromatography over silica gel (60-120 mesh, stationary phase) and eluted with solvents (mobile phase) of varying polarity, starting initially with petroleum ether, followed by combination of petroleum ether: ethyl acetate ranging from 95:5 then increasing 5 parts of ethyl acetate each time. Polarity of solvent system was further increased by adding 5 parts of Chloroform to 95 parts of ethyl acetate and increasing the Chloroform part in multiples of 5. Further, polarity was increased by eluting the column with the combination of Chloroform: Methanol ranging from 95:5 and increasing concentration of methanol in multiples of 5 to give a total of 318 fractions of 250 ml each. Fractions were dried on rotary vacuum evaporator and subjected to TLC.

Fractions 80-96 eluted in chloroform: ethyl acetate:: 1:1 showed similar pattern on TLC giving a major single spot, fractions 110-119 eluted in $CHCl_3$: methanol:: 4:1 showed similar pattern on TLC giving major single spot, fractions 139-156 eluted in $CHCl_3$: Methanol:: 3:2 showed similar pattern on TLC giving major single spot, fractions 229-256 eluted in $CHCl_3$: Methanol:: 1:4 showed similar pattern on TLC giving a major single spot and fractions 310-316 eluted in methanol showed major single spot on TLC. The spots were analyzed using iodine vapors and ceric ammonium sulfate as visualizing reagents and TLCs were monitored under UV cabinet. Fractions showing similar pattern were pooled, dried, purified on preparative TLC and recrystallized giving a total of five pure compounds namely Rhein, Emodin, Chrysophanic acid, Catechin and Luteolin and their melting points were recorded further which were subjected to spectroscopical analysis (Fig.1).

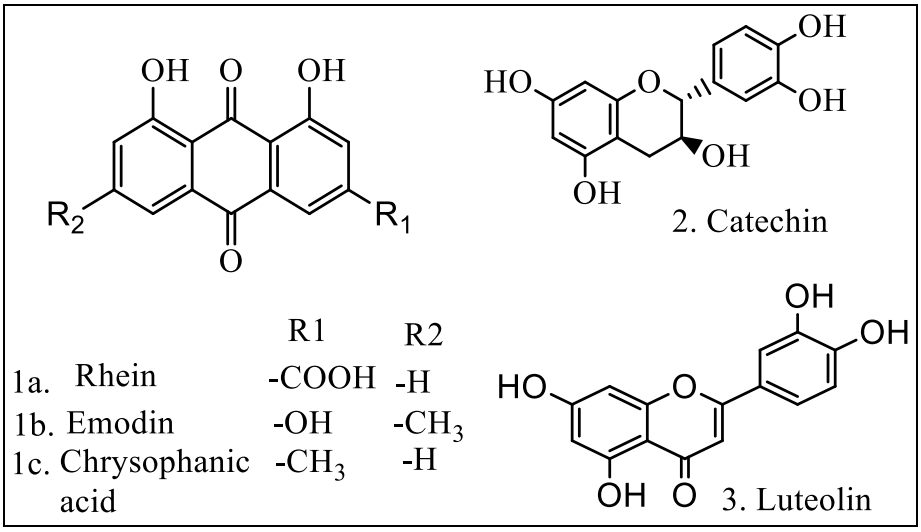

Fig 1: Structures of compounds isolated

***In-Vitro* Assessment of Anti-Oxidant Activity**

Hydrogen Peroxide Scavenging Assay

The ability of plant extracts (containing a large number of phenolics) to scavange H_2O_2 was estimated according to the method of Ruch *et al*. described by Alam *et al*. [27-28]. A 40mM solution of Hydrogen Peroxide was prepared in 50mM phosphate buffer (pH 7.4) whose concentration was determined from Hydrogen peroxide calibration curve by measuring absorbance at 230 nm using UV-VIS spectrophotometer. The methanol extract samples of different aerial parts of *C. fistula* were prepared at concentrations varying from 10, 20, 30, 40, 50 mg/ml by dissolving them in distilled water and mixture of hydrogen peroxide and phosphate buffer was added. The absorbance of the reaction mixture at 230 nm was determined after 10 minutes. The percentage of Hydrogen Peroxide Scavenging by the extracts was calculated as follows:

% scavenged (H_2O_2) = $[(A_c - A_s)/A_c] \times 100$

Where A_c is the absorbance of the control (without test sample) and A_s is the absorbance of the test sample. All experiments were performed in triplicates.

Determination of the Reducing Power

Previous reports show that the reducing power was associated with anti-oxidant activity which was due to the presence of anthraquinones, flavonoids and other polyphenolic compounds. For determination of reducing potential of methanol extracts of bark, leaves and pods of *C. fistula*, method described by Oyaizu was applied [28-30]. Substances having reduction potential can react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}). When ferric-chloride ($FeCl_2$) was added to reaction mixture, the potassium ferrocyanide (Fe^{2+}) reacts with it and produces a ferric-ferrous complex which can be spectrophotometrically measured at 700 nm. Various concentrations of plant extracts ranging from 10µg-50µg (10, 20, 30, 40 and 50µg) were prepared in methanol. Mixture of 0.2 M Phosphate Buffer (5ml, pH 6.6) and plant extract was added to 1% potassium ferricyanide (5ml) and incubated for 20 min at 50 °C. 5ml of 10% trichloroacetic acid (TCA) solution was added to the incubated reaction mixture and centrifuged for 10 min at 3000g. Supernatant (5ml) was mixed with distilled water (5ml) and 1% ferric chloride (1ml). Absorbance of the resulting reaction mixture was measured at 700nm. Increase in the absorbance indicated increase in reducing power which is associated with anti-oxidant activity. All the tests were carried out in triplicates.

Results

Methanol extract of pods of *C. fistula* contains rhein, emodin, chrysophanic acid, catechin and luteolin that were isolated and identified by using various chromatographic and spectroscopical techniques (Fig. 1).The experimental data of isolated compounds is as under:-

Rhein: Yellowish orange solid, Melting Point- 328 °C, Molecular Formula [$C_{15}H_8O_6$], IUPAC name- 1,8-dihydroxyanthraquinone-3-carboxylic acid. ^{1}H-NMR (400 MHz, DMSO) :- δ 7.34 (d, 1H, J = 8.58 Hz, H-5), 7.62 (m, 1H, H-6), 7.65 (s, 1H, H-4), 7.76 (d, 1H, J = 8.17 Hz, H-7), 8.02 (s, 1H, H-2), 11.43 (s, 1H, -OH), 11.81 (s, 1H, -OH).

Emodin: Orange solid, Melting Point- 236ºC, Molecular Formula [$C_{15}H_{10}O_5$], IUPAC Name- 6-methyl-1,3,8-trihydroxyanthraquinone. ^{1}H-NMR (400 MHz, DMSO):- δ 2.58 (s, 3H, -CH_3), 6.78 (s, 1H, H-2), 7.45 (d, 1H, *J*= 2.68 Hz, H-7), 7.91 (d, 1H, *J*= 2.68 Hz, H-5), 7.99 (s, 1H, H-4), 8.31 (s, 1H, -OH), 12.03 (s, 1H, -OH), 12.18 (s, 1H, -OH).

Chrysophanic Acid: Yellow solid, Melting Point- 324 °C, Molecular Formula [$C_{15}H_{10}O_4$], IUPAC name- 1,8-dihyrdoxy-3-methyl-9,10-anthraquinone. ^{1}H-NMR (400 MHz, DMSO):- δ 2.51 (s, 3H, -CH_3), 7.06 (s, 1H, H-2), 7.14 (s, 1H, H-4), 7.41 (d, 1H, J = 8.0 Hz, H-5), 7.58 (m, 1H, H-6), 7.82 (d, 1H, J = 8.0 Hz, H-7), 11.55 (s, 1H, -OH), 11.68 (s, 1H, -OH)

Catechin: Pale yellow solid, Melting point- 181 °C, Molecular Formula [$C_{15}H_{14}O_6$], IUPAC name- 3′,4′,5,7-tetrahyroxyflavan-3-ol. ^{1}H-NMR (400MHz, DMSO):- δ 2.38 (dd, 1H, H-4a), 2.70 (dd, 1H, H-4e), 4.43 (d, 1H, J = 7.91 Hz, H-2),4.61 (m, 1H, H-3),5.55 (d, 1H, J= 2.51 Hz, H-6), 5.77 (d, 1H, J= 2.51 Hz, H-8), 6.71 (dd, 1H, H-6′), 6.79 (d, 1H, J = 1.81 Hz, H-2′), 6.89 (d, 1H, J = 8.21 Hz, H-5′) and 8.16 (m, 5H, -OH).

Luteolin: Yellow solid, Melting point- 259 °C, Molecular formula [$C_{15}H_{10}O_6$], IUPAC name- 3′,4′,5,7-tetrahydroxyflavone. ^{1}H-NMR (400 MHz, DMSO):- δ 6.14 (s, 1H, H-6), 6.36 (s, 1H, H-8), 6.67 (s, 1H, H-3), 6.81 (d, 1H, J= 8.91 Hz, H-5′), 7.03 (d, 1H, J= 2.16 Hz, H-2′), 7.28 (m, 1H, H-6′) and 8.07 (m, 4H, -OH).

Hydrogen Peroxide Assay

The ability of the plant extracts to scavenge hydrogen peroxide may be attributed to their phenolic contents that can donate electrons to hydrogen peroxide, thus neutralizing it to water. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removal of H_2O_2 from food systems is an important issue.

The scavenging activity of methanol extract of bark, leaves and pods of *C. fistula* at different concentrations is shown in Fig. 2. The results clearly depicts that all the extracts can scavenge H_2O_2 to good extent. The scavenging potential of H_2O_2 increases with the increase in the concentration for all the aerial plant parts. At 30mg/ml, the percentage inhibition is above 50% for all the methanol extracts of aerial parts of the plant. At 10mg/ml, 20mg/ml concentration, all the methanol extracts except that of leaves exhibit more than 50% scavenging activity. The pods showed good scavenging activity at almost all the concentrations but at 40mg/ml and 50mg/ml it almost scavenged H_2O_2 completely indicating these to be a good antioxidant. The inhibiting percentage of methanol extracts of all the parts is in the following order: pods > bark> leaves.

Reducing Power Assay

The higher value of absorbance at 700 nm indicates stronger reducing power of the samples. The reduction potential of different parts of *C. fistula* viz. bark, leaves and pods and that of L-ascorbic acid at various concentrations is illustrated in Fig. 3. At low concentration, all the methanol extracts of different parts of the plant have shown good reducing power as compared to that of standard i.e. L-ascorbic acid, but at high concentration, reducing power of extracts is less as compared to L-ascorbic acid. The reducing powers of the samples are in the following order: pods > bark > leaves. The reducing power of pods of *C. fistula* is better than bark and leaves and comparable with L-ascorbic acid at every concentration indicating it to be a good reducing agent as compared to bark and leaves. There is continuous increase in the absorbance of methanol extracts of the all the parts of *Cassia fistula* with the increase in its concentration which has same pattern as that of L-ascorbic acid. Present investigation reveals that the reducing powers of the methanol extracts of all the parts of *C. fistula* also increases with the increase in their concentrations. So, it can be concluded that all the parts of the plant exhibited increase in their reducing potential in concentration dependent manner.

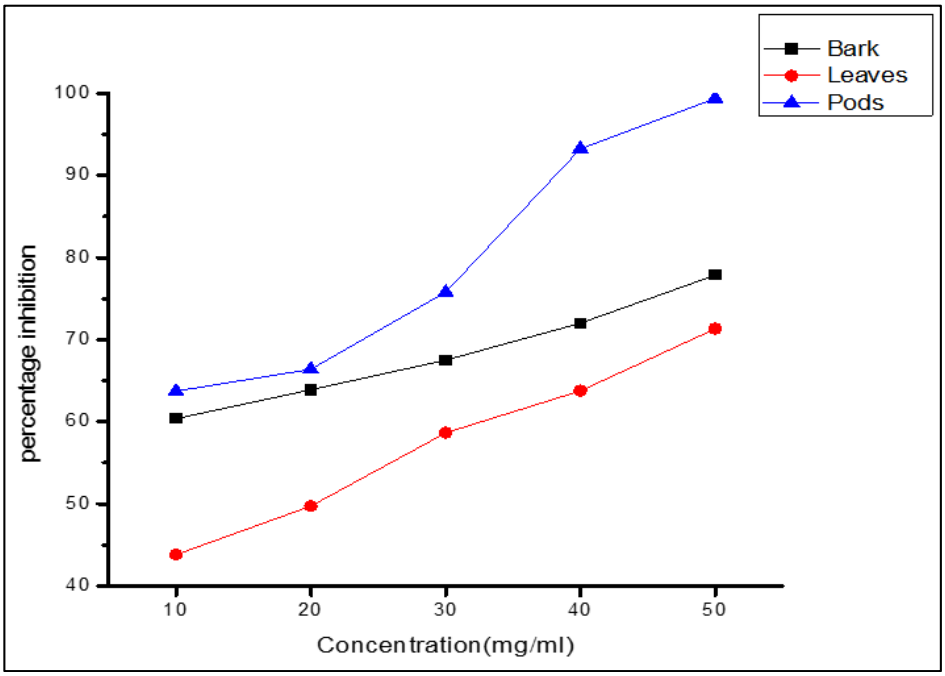

Fig 2: Percentage H_2O_2 Scavenging activity exhibited by methanol extracts of bark, pods and leaves at different concentrations

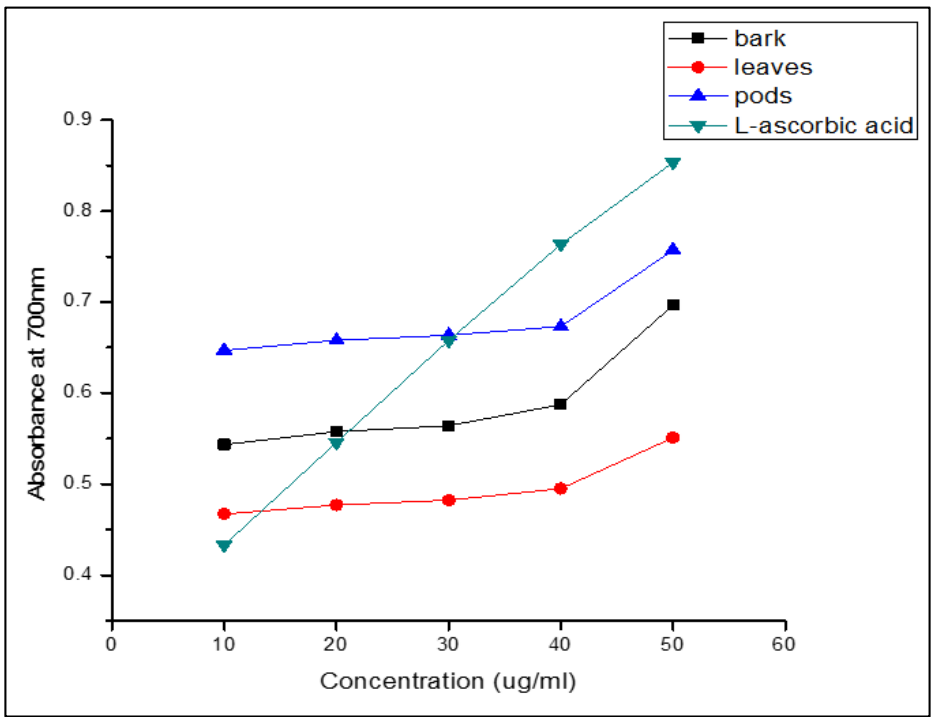

Fig 3: Reducing potentials of extracts of bark, leaves and pods of *C. fistula* at different concentrations.

Discussion

The results of the H_2O_2 scavenging assay and reducing power assay shows that bark, leaves and pods of *C. fistula* possesses good free radical scavenging potential against H_2O_2 and reducing potential because all the aerial parts (bark, leaves and pods) have shown positive results for the bioactivities employed, indicating these to be good anti-oxidants. The maximum scavenging potential against H_2O_2 and reducing potential was displayed by the pods of *C. fistula.* As a result, methanol extract of *C. fistula* pods was taken for isolation of secondary metabolites targeting anthraquinones, flavonoids and tannins using chromatographic techniques which results in isolation of pure compounds namely Rhein, Emodin, Chrysophanic acid, Catechin and Luteolin whose structures were characterized using various spectroscopic techniques and comparing them with Literature [6, 7, 31].

Rhein: The ^{1}H-NMR spectrum shows single peaks at δ11.81 (s) and 11.43 (s) attributed to hydrogen of hydroxyl group attached at position C-8 of ring A and C-1 of ring C respectively of the anthraquinones moiety. Singlet at δ8.02 corresponds to H -2 which is downfield due to deshielding effect of –OH group and –COOH groups attached to neighouring carbons at C-1 and C-3 respectively. Singlet at δ7.65 corresponds to H-4 of aromatic ring C showing downfield shift due to the presence of neighbouring –COOH group. Multiplet at δ7.62 corresponds to H-6 and doublet at δ7.76 and δ 7.34 corresponds to H-7 and H-5 of ring A respectively.

Emodin: The ^{1}H-NMR spectrum show peaks at δ12.18 (s) and 12.03 (s) that correspond to hydrogen of hydroxyl group attached at C-8 and C-1 of rings A and C respectively. Singlet at δ8.31corresponds to hydrogen of hydroxyl group attached at C-3. Singlet at δ7.99 and 6.78 corresponds to H-4 and H-2 of ring C which is downfield due to the presence of –OH group as neighboring group at C-3 and C-1 respectively. Doublets at δ7.91 and 7.45 corresponds to H-5 and H-7 of ring A respectively. Singlet at δ2.58 corresponds to three equivalent hydrogen of methyl group attached at C-6 of ring A.

Chrysophanic Acid: The ^{1}H-NMR spectrum shows peaks at δ11.68 (s) and 11.55 (s) that corresponds to hydrogen of hydroxyl group attached at C-1 and C-8 of rings C and A respectively. Singlet at δ2.51 corresponds to three hydrogens of methyl group attached at C-3 of ring C. Singlets at δ7.06 and 7.14 correspond to H-2 and H-4 of ring C respectively. Doublets at δ7.41 and 7.82 corresponds to H-5 and H-7 of ring A respectively. Multiplet at δ7.58 corresponds to H-6 of ring A.

Catechin: The ^{1}H-NMR spectrum shows doublet at δ4.43 that corresponds to H-2 of ring B. Doublet of doublet at δ2.38 and 2.70 corresponds to H-4a and H-4e respectively of same carbon and splitting in signal is observed due to different proton environment at C-4 and C-3. Multiplet at δ4.61 corresponds to H-3 of ring B and splitting in signal is due to different proton environment at neighbouring carbons i.e. C-2 and C-4. Doublets at δ5.55 and 5.77 correspond to H-6 and H-8 of ring A. Doublets at δ6.79 and 6.89 corresponds to H-2′ and H-5′ of ring C respectively. Doublet of doublet at δ6.71 corresponds to H-6′ of ring C.

Luteolin: The ^{1}H-NMR spectrum shows singlets at δ6.14 and 6.36 due to H-6 and H-8 of ring A respectively. Singlet at δ6.67 corresponds to H-3 of ring B. Doublets at δ6.81 and 7.03 corresponds to the hydrogen H-5′ and H-2′ respectively attached to ring C. Multiplet at δ7.28 corresponds to H-6′ of aromatic ring C.

Conclusion

The present study provides a method for extraction, isolation and identification of pure compounds from aerial parts *i.e.* bark, leaves and pods of *Cassia fistula.* Simple Chromatographic techniques viz. Column Chromatography, TLC and preparative TLC, employed for separation of different bioactive fractions resulted into isolation of five major compounds namely Rhein, Emodin, Chrysophanic acid, Catechin and Luteolin. Melting points and ^{1}H-NMR spectrum confirmed the structures of isolated phenolic compounds. The *in-vitro* anti-oxidant activity evaluation of methanol extract of bark, leaves and pods shows good scavenging and reducing potential of *C. fistula* plant in H_2O_2 scavenging assay and reducing power assay proving it to be a good anti-oxidant. As the anti-oxidant potential is directly linked to the phenolic content [29], so we can conclude that aerial parts *i.e.* barks, leaves and pods of *C. fistula* are rich in phenolic compounds which are accountable for their anti-oxidant potential. Methanol extracts of the aerial parts of *C. fistula* exhibiting reducing and scavenging potential is in the following order: pods > bark > leaves.

So, it is concluded that pods exhibit much elevated anti-oxidant potential as compared to bark and leaves, which is due to the presence of anthraquinones (Rhein, Emodin, Chrysophanic acid), tannin (Catechin) and flavonoids (Luteolin).

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