Anthraquinone rich *Cassia fistula* pod extract induces IFIT1, antiviral protein

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*Cassia fistula* L., an Indian Laburnum has therapeutics significance in human healthcare since ancient days. Its pod is known to rich sources of anthraquinones. Anthraquinone compounds have antiviral as well as immune modulatory properties. Human IFIT1 protein (Interferon induced protein with tetratricopeptide repeats 1) is a crucial component of innate immunity. The expression of IFIT1 protein normally remains silent but strongly induced after interferon’s (IFNs) treatment or viral infections. IFIT1 protein indeed has broad spectrum of antiviral activity by blocking different stages of viral replication, translation and assembly of new viral proteins. Modulation of IFIT1 protein expression may be useful in the treatment of viral diseases. The aim of present study is to identify *Cassia fistula* pod rich with anthraquinones extract (CF) to induce IFIT1 antiviral protein. Therefore, CF is extracted by soxhlet extraction process. HT1080 cells were used to study the expression of IFIT1 with CF extract. The IFIT1 protein induction in cells was assessed by Western blot and RT-PCR. The results showed that, CF extract significantly induced IFIT1 antiviral protein expression suggesting anthraquinones as potential agonistic compounds for inducing innate immune system to treat viral infections.

**Keywords:** Medicinal plant, Innate immunity, Interferon, Viral infections

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*Cassia fistula* L., a semi-wild Indian labrum and furthermore recognized as the Golden Shower has therapeutics significance in human healthcare since old circumstances\(^1\). *Charaka* and *Sushruta Samhita* has also reported the usages of *Cassia fistula* as a part of the treatment for different diseases in ancient India\(^2\). It has been broadly utilized as a part of various conventional prescriptions including *Ayurveda*, Chinese and *Unani* for the treatment and avoidance of diseases\(^3\). In *Ayurvedic* medicine, *Cassia fistula* is used against various diseases such as leucoderma, pruritus, and haematemesis\(^4\). The antifungal and antibacterial effect of *Cassia fistula* has also been reported\(^5\)-\(^7\). Some parts of plants, for example, stem, leaf, root and pod contain distinctive chemical constituents, and such constituents have demonstrated therapeutic potential in human healthcare\(^8\) such as antitumor\(^9\), antioxidant\(^7\), hypoglycemic\(^10\), inflammatory diseases, rheumatism, ulcers, skin diseases and anorexia activities\(^11\).

*Cassia fistula* pods were broadly used as a traditional medicine for antipyretic, diuretics and laxatives\(^12\). In some studies, the ethanol extract of *Cassia fistula* pod showed inhibition against (Ranikhet disease virus (RDV), Vaccinia virus\(^13\). Moreover, an aqueous extract of *Cassia fistula* pod inhibits dose-dependent anti-bovine rhinotracheitis virus activity\(^14\). The active principles of *Cassia fistula* pod are anthraquinones, in which rhein, aloin, emodin, sennosides, fistulous acid, aolin, 3-formyl-1-hydroxy-8-methoxyanthraquinone, chrysophanol and aloe-emodin are significant components\(^15\)-\(^19\). These are in aglycone and glycoside frames. Many studies have indicated that the anthraquinone compounds have antiviral and immune modulation properties, such as modulation of humoral immunity\(^20\), induces proliferation of CD4\(^+\) T lymphocytes of human and secretion of IL10\(^21\), inactivation of enveloped viruses\(^22\), antiretroviral activities\(^23\), antiviral activity against influenza A virus\(^24\), polio virus\(^25\) and hepatitis B virus replication\(^26\).

In the recent years, several groups have recognized novel genes downstream signaling of type I interferon to inhibit infection of viruses\(^27\). These genes are named interferon-stimulated genes (ISGs) or interferon-induced protein with tetratricopeptide...
repeats (IFIT). This ISGs gene shows a broad spectrum of antiviral activity. Among the ISGs, IFIT1 (ISG56) protein has gained enormous attention in antiviral activity. IFIT1 protein rapidly induced by virus-infected cells with interferon-dependent or interferon independent pathways. IFIT1 protein has tetratricopeptide repeats (TPRs) with distinctive helix-turn-helix motifs, which play important role in protein-protein and protein-RNA interactions. Because of structural adaptability of IFIT1 protein shows a diverse biological effect that inhibits different phases of the viral lifecycle including virus entry, assembly, translation and viral replication. A couple of examples are, inhibition of replication of Human Papillomavirus (HPV) by binding to its helicase E1 protein, restraint of replication of the Japanese encephalitis (JEV) infection by binding to 5’ capped 2’-O unmethylated RNA. Inhibition of Hepatitis C virus (HCV) translation initiation by binding to elongation initiation factor, inhibition of viral mRNAs translation which lacks N7- and 2’-O-methyltransferases, and also exhibits antiviral activity against influenza A virus and vesicular stomatitis virus.

Thus, IFIT1 protein indeed has a broad spectrum of antiviral activity. Modulation of IFIT1 expression may offer novel approaches for the treatment of viral diseases. Several medicinal plants could offer a rich asset for the medication disclosure against viral infections. *Cassia fistula* pod is rich sources of anthraquinone secondary metabolites. Many studies have indicated that the anthraquinone secondary metabolites have antiviral and immune modulation properties. In the current study, we aimed to test the ability of *Cassia fistula* pod extract rich with anthraquinones to induce IFIT1 antiviral protein.

**Material and methods**

**Chemicals**

Dulbecco’s modified Eagle’s minimum essential medium (DMEM), pen-strep solution and fetal bovine serum (FBS), were obtained from Gibco-Thermo fisher scientific. Monoclonal antibodies of IFIT1 (Sc-134948), β-actin (Sc-47778), and peroxidase-conjugated secondary antibody (Sc-2004) were bought from Santa Cruz Biotechnology, Inc. MTT, rhen obtained from Sigma–Aldrich. ECL plus detection kit procured from GE Healthcare Biosciences. RT-PCR kit was obtained from Affymetrix, USB. IFIT1 and β-actin primers were synthesized from Integrated DNA Technologies.

**Plant material**

*Cassia fistula* pods were collected from University of Hyderabad campus, Telangana, India. The plant was authenticated by Prof. Ajmeera Ragan, Department of Botany, Kakatiya University, India. The voucher specimen (KUW: 1333) deposited the herbarium of Kakatiya University, India.

**Preparation of anthraquinone crude extract**

*Cassia fistula* pod along with seed was powdered (100 g) and extracted with methanol (500 mL) for 24 h using Soxhlet apparatus. The extract was filtered and evaporated to dryness under reduced pressure. To separate anthraquinone from the methanol extract, method established by Stephen Chao Yung Su was used. In this method, chloroform and ethyl acetate solvents were used to separate hydroxyanthraquinones. The methanol crude extracts obtained above was macerated with ethyl acetate solvent at 24 h. Ethyl acetate solvent liberated anthraquinones from the extract. The maceration mixture was air dried and extracted with chloroform until free of anthraquinones. The anthraquinone extraction monitored by Borntrager’s reaction. The anthraquinone extracted chloroform layer was concentrated under reduced pressure using rotary evaporator. The extract was stored at 4 °C. Methanol and chloroform extracts were weighed and their percentage yield was calculated.

**Identification of Anthraquinones**

Borntrager’s reaction was used to identify the presence of anthraquinone in the extract. Briefly, 2M HCl was added to the extract and boiled in a hot water bath for 15 min, later the test tube containing boiled extract was cooled and filtered. The filtrate mixture was extracted with chloroform, further separated and mixed with dilute ammonia solution. The upper layer becomes pink in color. The procedure for quantitative analysis of total anthraquinone in the chloroform extract was measured by UV-visible spectrophotometric as described earlier.

**Thin layer chromatography (TLC) and LC-MS analysis of anthraquinone extract**

Chromatographic analysis of anthraquinone extract was performed by TLC on a pre-coated aluminum plate of silica gel (12 x 6 cm) using petroleum ether:ethyl acetate:glacial acetic acid (90:10:6) as the mobile phase. 10 % alcoholic potassium hydroxide (KOH) solution was used to visualize the spots of
anthraquinones. Anthraquinone show pink-red spots. The hRF of anthraquinone components were determined by comparing with the standard hRF value of rhein\textsuperscript{18}. Further, the extract was evaluated by LC-MS (Liquid chromatography-mass spectrometry).

Cell culture
HT1080 cells (Human fibrosarcoma cell line) were procured from National Center for Cell Science (NCCS), Pune, India. HT1080 cells were maintained in complete DMEM media added with FBS (10 %) and pen-strep solution (100 IU/mL), at 37 °C in an atmosphere of CO\textsubscript{2} (5 %).

MTT assay to measure the cytotoxic dose of extract
The in vitro cytotoxicity assay was carried with the CF extract to find out the MNTD (maximum-non toxic dose) of HT1080 cells. Briefly, these cells cultured in 96-well cell culture plate. After 12 h different concentration of the CF extract prepared in complete media was added to the cultured cells. The 96 microtitreplates were incubated at 37 °C with 5 % CO\textsubscript{2} in an incubator for a period of 24 h and 48 h. After the CF extract treatment, the media was replaced with 100 µL new complete DMEM media. 20 µL of MTT (5 mg/mL) solution were added to each microtitre-96 well, further incubated at 37 °C for 4 h. After 4 h incubation media was removed carefully and added 150 µL of DMSO. MTT formazan product absorbance was determined by ELISA plate reader at 590 nm with the reference filter set to 620 nm.

Western blotting for IFIT1 protein
To test the effect of CF extract to induce IFIT1 expression in HT1080, CF extract treated and untreated cells were collected by centrifugation. The cell pellets were re-suspended in RIPA lysis buffer and incubated for 30 min. The protein concentration was ascertained by BCA Protein assay kit. 50 µg of CF extract treated and untreated protein was electroblotted onto a nitrocellulose membrane, further separated on 10 % SDS-PAGE. The nitrocellulose membrane was blocked by 1X TBST containing 5 % non-fat dry milk at RT for 1 h. The membrane was incubated with IFIT1 antibody with 1:4000 dilutions and for β-actin 1:5000 dilution at room temperature for 2 h. The later nitrocellulose membrane was washed with TBST (3 times) followed by incubation with secondary antibody at room temperature for 1 h. Nitrocellulose membrane blots were developed by ECL plus Western blot detection solutions. Fiji Image analysis software was used to determine the band intensities.

RT-PCR for IFIT1 mRNA
Total CF extract treated and untreated cellular RNA was isolated using Trizol RNA isolation method. Total cDNA was prepared from the isolated RNA sample using two steps RT-PCR kit. PCR experiment was performed to detect IFIT1 mRNA and β-actin mRNA as an internal control. The volume of PCR reaction was 25 μL containing: 1.25 unit of TaqDNA polymerase, 0.2 mM of dNTPs, 1X reaction buffer, and 0.4 μmol of forward and reverse primers. The PCR amplification cycles were performed for IFIT1 and β-actin (initial denaturation at 95 °C for 3 min, denaturation 95 °C for 30 sec, annealing at 56 °C for 1 min, and extension at 72 °C for 30 sec). The PCR primers of IFIT1 (Forward 5’ – TAGCCAAACATGTCCCTCACAGAC-3’; Reverse 5’ – TCTTCTACCAGCTGGTTCATGC-3’) and β-actin (Forward 5’ –GCTCGGGCTGTCAGAA-3’; Reverse 5’ –AGGATCTTCTAGGGTAGT-3’) was used in this study. The PCR reaction samples were electrophoresed on agarose gel. The PCR amplified bands visualized by EtBr (ethidium bromide) staining under UV gel doc. Fiji Image analysis software was used to determine the band intensities.

Statistical evaluation
Simple linear regression analysis was performed to calculate the mean values of anthraquinones content in Cassia fistula pod by using GraphPad Prism software v. 7. The method of relative quantification was used for analyzing the results of western blot and PCR with respect to their blots.

Results
Extraction and identification of Anthraquinones
In order to identify CF inducing IFIT1 protein, we have initially extracted Cassia fistula pod with MeOH. The yield of methanol crude extract from 100 g Cassia fistula pod was 15 g or 15 %. Further, MeOH extract was further subjected to ethyl acetate and chloroform extraction. The yield of chloroform extract from 15 g of methanol crude extract was 1 g or 6.6 %. Bornträger’s reaction was used to monitor anthraquinone extraction process. The extract of CF gave a positive result for anthraquinones in Bornträger reaction and showed four spots on the TLC.
plate. The UV-visible spectrophotometric method used to calculate the total content of anthraquinones in CF extract. The relationship was taken within the concentration range of 10-50 µg/mL of rhein standard with correlation coefficient \( r = 0.9932 \). The representative linear equation for rhein was \( y = 0.0434x - 0.0025 \). The total content of anthraquinones calculated as compared to rhein was 36 µg/mL. In LC-MS analysis, identified anthraquinones are rhein, aloe-emodin, chrysophanol, emodin, physcion and cassialoin. In LC-MS analysis indicating RT (retention time) of the rheinis 12.8 and area percentage 15, alo-emodin RT is 9.9 and area percentage 5.42, chrysophanol RT is 9.1 and area percentage 50.16, cassialoin RT is 6.2 and area percentage 2.9, physcion RT is 10.4 and area percentage 8.32, emodin RT is 12.6 and area percentage 1.07.

**Maximum nontoxic dose (MNTD) of extracts**

The maximum non-toxic dose of extracts in HT1080 cells was determined prior to IFIT1 expression analysis. MTT assay showed that treatment of HT1080 cells with CF extract at different concentrations (25 µg/mL to 200 µg/mL) for 24 and 48 h. In 24 h treatment, CF extract did not show significant cytotoxicity (Fig. 1). The cytotoxicity results indicate that the concentrations used for IFIT1 expression analysis could not be attributable to non-specific cytotoxicity.

**Effects of CF extract on IFIT1 protein expression**

Western blot analysis was carried out to check the result of CF extract on HT1080 cells for IFIT1 protein expression. HT1080 cells were mixed with various concentrations (0, 10, 40, 80 µg/mL) of CF extract. Interestingly, CF extract significantly expressed IFIT1 in HT1080 cells in a concentration of 40 and 80 µg/mL (Fig. 2).

**Effect of CF extract on IFIT1 mRNA expression**

To examine the effects of CF extract on the expression of IFIT1 mRNA level, the RT-PCR was performed. Poly I:C used as positive control for expression of IFIT1 mRNA. HT1080 Cells were treated with poly I:C (50 µg/mL) and CF extract (80 µg/mL) for 10 h to check induction of IFIT1 mRNA. After 10 h incubation, total cellular RNA was extracted from treated, untreated HT1080 Cells; prepared cDNA was amplified by PCR using IFIT primers, β-actin mRNA used as an internal control.

Fig. 3 shows that CF extract induces IFIT mRNA expression significantly as similar to that of Poly (I:C). While the extract had no effect on the expression of β-actin.

**Discussion**

In the present study, we tested *Cassia fistula* pod for induction of a potential antiviral protein, IFIT1. *Cassia fistula* pod is known to have high anthraquinone compounds which are known to have anti-inflammatory as well as immune modulatory properties but a scientific evaluation was not carried out yet. Research work from many laboratories shown that IFIT has a direct antiviral property where it interferes with different stages of the viral replication.
process. The expression of IFIT is not detected in
resting cells but induces > 200 folds upon virus
infection or interferon treatment. So induction of IFIT
protein is a positive feedback towards antiviral state
for cells. In this notion, we started our work in
evaluating CF extract for IFIT1 gene induction
through PCR as well as western immunoblotting. The
CF extract induces the expression of IFIT1 in a
dose-dependent manner at the protein level using specific
IFIT1 antibody. The protein expression data was
further supported by mRNA expression data where
CF extract induces IFIT1 expression was comparable
to poly I:C induction. Further, we confirmed the
presence of anthraquinone compounds in the CF
extract through thin layer chromatography and LC-
MS analysis. Anthraquinone compounds have been
shown to have direct antiviral properties against many
viruses; for example, human immunodeficiency
virus. Where anthraquinones inhibit viral reverse
transcriptase activity and human cytomegalovirus. Overall, our present study confirms CF extract to have
IFIT1 induction ability which predicts and confirms
ethnically known medicinal properties of CF as
antiviral. Further studies are needed to delineate the
specific actions of anthraquinone found in CF to
inhibit a specific viral replication/multiplication.

Conclusion
Cassia fistula broadly used as a tradition for
antipyretic and antiviral activity. The mode of action
of antiviral activity has been studied through
inactivating viruses. The current study revealed that
the Cassia fistula pod rich with anthraquinones
extract (CF) significantly induced IFIT1 antiviral
protein. The IFIT1 induction of the CF extract as
showed by the Western blot and RT-PCR. Therefore,
the present study confirms CF extract to have IFIT1
protein induction might give a very important insight
into the development of anti-viral therapeutic from the
Cassia fistula anthraquinones.

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