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Antioxidant and Anti-Apoptotic Activities of Phytochemically Validated Fruit Extract of *Solanum xanthocarpum* in Primary Chondrocytes

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Abstract

The chondrocyte death may contribute in progression of osteoarthritis (OA). *Solanum xanthocarpum* (Family: Solanaceae) fruits were known for antioxidant activity. This study demonstrates that the phytochemically validated *Solanum xanthocarpum fruits* (SXF) extract has inhibitory activities on nitric oxide (NO) induced cell death and ROS formation in primary cultured chondrocytes. Chondrocyte death was induced by 1.5 mM of Sodium Nitroprusside (SNP). The Cell viability was measured by MTT assay and nuclear changes were observed by DAPI and Hoechst-PI. Antioxidant activity of SXF was demonstrated in H_2O_2 induced ROS generation in chondrocytes. Indomethacin (IM) (25μM), a NSAID was taken as positive control. Phytochemical analysis revealed the presence of flavonoids, anthraquinone glycosides, steroids, alkaloids, terpenoids and tannins. SXF significantly reduces the cell death induced by SNP in a dose dependent manner. The fluorescent photomicrograph of DAPI, Hoechst-PI and ROS also revealed the decreased rate of apoptosis in a dose-dependent manner. This study suggests that SXF shows anti-apoptotic and antioxidant activity in chondrocytes.

Keywords: Apoptosis, Chondrocytes, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), Osteoarthritis, Phytochemicals

1. Introduction

Osteoarthritis is associated with the breakdown and ultimate loss of articular cartilage of joints[1] and is commonly occurs among the elderly population in the world[2]. Several etiological risk factors like age, gender, trauma, overuse, genetics and obesity are associated with pathophysiologic processes that contribute disease progression[3]. In the pathological condition the cells of articular joints are subjected to complex environmental control. In addition to various cytokines, growth factors, and mechanical stimuli, reactive oxygen specie (ROS) contributes in pathological condition. Therefore, a functional change in chondrocytes of articular cartilage is related to the progression of OA[4]. Overproduction of oxidants (reactive oxygen species and reactive nitrogen species) in the human body is responsible for the pathogenesis of some diseases. Nitric Oxide (NO) and superoxide anion (O_2^-) are the main ROS produced by chondrocytes[5]. ROS like superoxide anion (O_2^-), Hydrogen Peroxide(H_2O_2), and hydroxyl radicals (OH^-) are the byproduct of aerobic metabolism[6] and are associated with principal oxidative stress molecules. The enzyme complex NADPH catalyzes the reduction of molecular oxygen to superoxide anion radicals[4]. The production of NO is stimulated by various cytokines including interleukin (IL)-β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and lipopolysaccharides (LPS), and inhibited by Transforming growth factors (TGF)-β, IL-4, IL-10 and IL-13[7–9]. It is believed that NO is an important mediator of dedifferentiation and apoptosis of chondrocytes in arthritic cartilage[10].

Non-steroidal anti-inflammatory drugs are commonly used drugs in the entire world for the treatment of osteoarthritis. Long-term use of these NSAIDs leads to significant side effects on liver, stomach, gastrointestinal tract and heart[11]. Therefore it becomes essential to explore alternative medi-

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cine derived from herbal plants with a potential drug that is effective in terms of both efficacy and safety. Medicinal plants provide a significant source of chemical compounds that have a great importance on the health of individual and community. There is wide diversity of chemical compounds that have been isolated from plant especially secondary metabolites that were shown to have anti-cancer, anlegesic, anti-inflammatory, anti-bacterial and including some other activities[12,13]. These phytochemicals include flavonoids, phenols and phenolic glycosides, saponins and cyanogenic glycosides, tannins, nitrogen compounds (alkaloids, amines, betalains), terpenoids etc[12]. Antioxidants have importance regarding reducing oxidative stress that could otherwise affect and damage biological molecules[14]. Bioactive components such as flavonoids are natural antioxidant due to its indigenous origin and have strong efficacy to scavenge free radicals[15].

Solanum xanthocarpum Schrad. Wendl. is commonly known as yellow-berried nightshade (Syn: *Solanum surattens Burm. F.; Solanum virginium Linn*) that belongs to family solanaceae. It is prickly diffuse bright green perennial herb, somewhat woody at the base. The stem is zig-zag with numerous branches. The berries are globular with green and white stripes when young but yellow when mature and surrounded by the enlarged calyx[16]. In Hindi, it is called Kantkari. Its other names are Choti Katheri, Kateli, Bhatkatiya and Bhachkatiya. It has been reported to occur in Ceylon and Malacca through South-East Asia, Malaya, Australia and Polynessia[17]. It is a wild plant mainly grown in Uttar Pradesh, Uttaranchal, Bihar, Punjab, West Bengal, Assam and other North-Eastern states[18]. It is a commonly used Ayurvedic medicine for treatment of asthma and bronchitis. Fruit juice of the plant is useful in treatment of sore throats and rheumatism, Decoction of the plant is used in gonorrhea, paste of leaves is applied to relieve pains, seeds act as expectorant in a cough and asthma, roots are expectorant and diuretic and useful in the treatment of catarrhal fever, coughs, asthma and chest pain[19].

This study is designed to evaluate the antioxidant and anti-apoptotic efficacy of phytochemically validated SXF extract in primary chondrocytes isolated from rat articular cartilage.

2. Material and Methods

2.1 Materials

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, 1:1 mixture), 2',7'-dichlorofluorescein diacetate (DCFH-DA) and Propidium Iodide (PI) were from Sigma-Aldrich Inc. St. Louis, USA. Fetal Bovine Serum (FBS), sodium pyruvate, Non-Essential Amino Acids (NEAA), sodium bicarbonate, L-glutamine, antibiotic solution (penicillin/streptomycin), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye, were all purchased from Himedia Laboratories Pvt. Ltd. Mumbai, India. Dimethyl Sulfoxide (DMSO), was from Merck Specialities Pvt. Ltd. Mumbai, India. All other reagents were of analytical grades.

2.2 Collection, Identification and Preparation of Plant Extract

The fruits of *Solanum xanthocarpum* were collected from roadsides in Gomti-Nagar and Kursi Road, Lucknow, India in month from September to February. The plant is identified by Prof. S. Lavania, Deparment of Botany, University of Lucknow, Lucknow. A reference of specimen (Voucher No. LWU-2016-4) has been deposited in the herbarium of Department of Botany, University of Lucknow, Lucknow.

The fresh plant material was collected, washed twice with double distilled water, then shade-dried and turned into powdered. The 95% ethanolic extract of plant was prepared with the help of Soxhlet apparatus (Borosil Glass Works Limited, India).

2.3 Phytochemical Screening

Before evaluating antioxidant and anti-apoptotic activity, the ethanolic extract of SXF was tested for the presence of phytoconstituents by standard biochemical tests for alkaloids, steroids, tannins, saponins and glycosides. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

2.3.1 Test for Alkaloids

About 15 mg of SXF extract was taken in a test-tube and stirred with 1% HCl (6 mL) on a water bath for 5 min and filtered. These filtrates were divided into three equal parts.

- **Dragendorff's Test:** To the first portion of the filtrate, 1 mL of Dragendorff's reagent (Potassium bismuth iodide solution) was added. Formation of an orange-red precipitate shows the presence of alkaloids.
- **Mayer's Test:** To the second portion of the filtrate, 1 mL Mayer's reagent (Potassium mercuric iodide solution) was added. A cream-colored precipitate indicates the presence of alkaloids.

- **Wagner's Test:** About 2 g Potassium iodide and 1.27 g iodine were dissolved in 10 mL distilled water and diluted to 100 mL with distilled water. To the third portion of the filtrate, a few drops of prepared solution were added. The appearance of a brown colored precipitate indicates the presence of alkaloids[20,21].

2.3.2 Tests for Steroids and Terpenoids

- **Salkowski Test:** About 100 mg of SXF extract was taken in a test-tube. Dissolve the extract in 2 mL of chloroform (2 mL) by shaking followed by the addition of 2 mL concentrated H_2SO_4 along the side of the test tube. The appearance of reddish-brown coloration of the interface indicates the presence of terpenoid[22].
- **Liebermann-Burchard Test:** About 100 mg of extract was shaken with chloroform in a test tube. A few drops of acetic anhydride was added to the test tube and boiled in a water bath, which is rapidly cooled in iced water. A 2 mL concentrated H_2SO_4 was added along the sides of the test tube. Formation of a brown ring at the junction of two layers and turning the upper layer to green indicates the presence of steroids while the formation of deep red color shows the presence of triterpenoids[21].

2.3.3 Test for Tannins

About 0.5 g of SXF extract was separately stirred with 10 mL distilled water and filtered. A few drops of 5% ferric chloride were added to test-tube. Black or blue-green coloration or precipitate indicates the presence of tannins[23].

2.3.4 Test for Saponins

About 5 g of SXF extract was separately shaken with 10 mL distilled water in a test tube. The formation of frothing, which remains persist on warming the test-tubes in a water bath for 5 min, indicates the presence of saponins[23].

2.3.5 Tests for Glycosides

- **Anthraquinone glycoside (Borntrager's Test):** To the 1 mL of SXF extract solution, 1 mL of 5% H_2SO_4 was added. The mixture was boiled in a water bath for 5 min and then filtered. The filtrate was then shaken with an equal volume of chloroform and kept to stand for 5 min. A 1 mL of dilute ammonia was shaken with the lower layer of chloroform. There is formation of rose pink to red-color of the ammoniacal layerthat indicate-santhraquinone glycosides[21].
- **Cardiac glycoside (Keller-Killiani Test):** About 0.5 g extract was shaken with 5 mL distilled water. A 2 mL glacial acetic acid containing a few drops of ferric chloride was added, followed by 1 mL of H_2SO_4 along the side of the test tube. The formation of a brown ring at the interface gives a positive result for cardiac glycoside and a violet ring may appear below the brown ring[22].

2.3.6 Tests for Flavonoids

- **Shinoda Test:** About 1 g of SXF extract was taken in test-tube and mixed with pieces of magnesium ribbon and concentrated HCl for few minutes. The appearance of pink color showed the presence of flavonoid.
- **Alkaline Reagent Test:** About 1 gm of SXF extract was taken in test-tube andmixed with 2 mL of 2.0% NaOH. The intense yellow color was produced that became colorless when 2 drops of diluted acid was added to this mixture showed the presence of flavonoids.

2.3.7 The Culture of Primary Chondrocyte Cells

The primary chondrocytes were isolated from knees of 2-3 days old rat pups. Isolated cartilage was transferred to phosphate buffer saline (PBS) with 500 U/mL penicillin and 500 µg/mL streptomycin. Then the cartilages were cut into small pieces, and subjected to digestion with 0.25% trypsin/EDTA and kept at 37°C, 5% CO_2 incubator for 30 min. The supernatant was centrifuged and resulting pellet was digested twice with 0.2% type II collagenase for 1 h each and kept in a CO_2 incubator and centrifuged at 1200 rpm for 6 min to obtain a final cell pellet. Cells were re-suspended in DMEM/F-12 complete culture medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and placed in 50 mL culture flask[24].

When the cells reached up to 80-90% confluency, the cell morphology was observed under phase contrast microscope (Nikon ECLIPSE Ti- S, Japan).

2.4 MTT Assay for NO Induced Cell Death

Chondrocytes were suspended in Chondrocyte Growth Medium at a density of 1×10^4 cells/mL and cultured in 96-well plates at 37^0C in 5% CO_2 for 1 day. After medium change with DMEM/F-12 supplemented with 100 U/mL penicillin, 100

μg/mL streptomycin, and 20 μg/mL gentamicin, chondrocytes were pretreated with 25 μM indomathacin (IM) SXF at concentrations (50, 100, 250 μg/mL) for 1 day. Cell death was induced by treatment of cells with 1.5 mM of SNP for further 24 h. The cell viability was evaluated with a soluble tetrazolium salt MTT [3-(4,5-dimethylthiazole-2-yl)- 2,5-diphenyltetrazorium bromide][25].

2.5 Nuclear Apoptosis Assay

4,6-Diamidino-2-Phenylindole-2-HCl (DAPI) binds dsDNA that provides a blue fluorescence when viewed under the ultraviolet light. Apoptotic cells are visualized as a small, condensed nucleus. The cells were seeded and treated for 24 h in 96-well plate in medium containing 10% FBS and 1% penicillin/streptomycin solution. Then, the different dose of SXF (50, 100, 250 μg/mL) and 25 μM IM was added to each well with complete media. After the treatment period, cells were exposed to SNP and further incubated for 24 h. The cells were washed with PBS and fixed in 4% PFA for 10 min. Subsequently, the cells were permeabilized with permeabilization buffer (3% PFA and 0.5% Triton X-100) and stained with DAPI. After the staining images were taken with the fluorescent microscope (Nikon Eclipse Ti-S, Japan)[26].

2.6 Hoechst-Propidium Iodide (PI) Double Staining

This dye is used to detect normal, apoptotic and dead cells in same culture well. Hoechst is used to stain chromatin of apoptotic cells with fluorescence than normal cells. The PI on the other hand is used to stain chromatin of dead cells. The staining procedure was according manufacturer's protocol (GenScript). The cells were treated with different concentrations of SXF (50, 100, 250 μg/mL) and 25 μM of IM. Further the cells were exposed to SNP for 24 h in CO_2 incubator. A 1 μL of Hoechst 33342/100 μL PBS was loaded in each well and incubated in CO_2 incubator for 10 min. After aspiration, a 100 μL of 1X buffer A mixed with PI was loaded. Plate was then incubated at room temperature in dark for 5 min. Cells were immediately visualized under inverted fluorescence microscope (Nikon, ECLIPSE Ti-Series).

2.7 DCFH-DA Staining for Reactive Oxygen Species (ROS)

ROS generation was assessed by 2',7'-dichlorofluorescein diacetate (DCFH-DA) dye. Chondrocyte cells were seeded in black bottom culture plate for 24 h and incubate the plate at 37 °C, 5% CO_2 maintained in the CO_2 incubator. Cells were then exposed to 20 μL (10 μM stock solution) of H_2O_2 for 24 h. Cells were treated with 25 μM of IM and SXF at concentrations 50, 100 and 250 μg/mL for 24 h in triplicate. The cells were further incubated with DCFH-DA dye (stock 10 mM) for 30 min. The reaction mixture was kept on the shaker for 10 min at room temperature in dark. Fluorescence intensity was measured with a multi well plate reader (Synergy H1 Hybrid Multi-mode microplate reader, BioTEK) at an excitation wavelength of 528 nm. Photomicrographs of another set of cells seeded in 96 wells plate were taken by fluorescence microscope (Nikon Eclipse Ti-S, Japan) to analyze intracellular fluorescence intensity of ROS production[26].

3. Results

3.1 Phytochemical Screening

The outcome through phytochemical screening shows that the whole SXF ethanolic extract contains flavonoids, anthraqui-

Table 1. Phytochemical validation of locally collected *Solanum xanthocarpum* fruit extract through standard biochemical tests

	Phytochemical Test	Investigation (Present/ Absent)
1	**Test for Alkaloids** (a) Dragendorff's Test (b) Mayer's Test (c) Wagner's Test	 + + +

Table 1 Continued

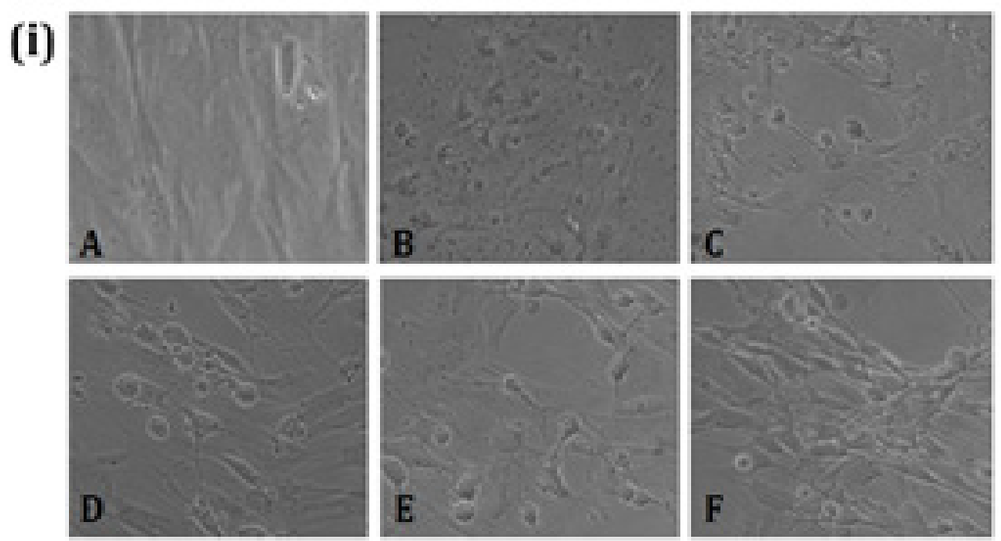
2.	**Tests for Steroids/terpenoids** (a) Salkowski test (b) Liebermann-Burchard Test	 + +
3.	**Test for Tannins**	+
4.	**Test for Saponins**	+
5.	**Test for Glycosides** (a) Anthraquinone Glycoside (Borntrager's Test) (b) Cardiac Glycoside (Keller-Killiani Test)	 + +
6.	**Test for Flavonoids** (a) Shinoda test (b) Alkaline reagent test	 + +

Present = (+) and Absence = (-)

none glycosides, steroids, alkaloids, terpenoids and tannins. The result has been demonstrated in Table 1.

3.2 Inhibition of NO induced cell death

The production of NO is an important component that involves in the pathogenesis of OA. We address, whether the given extract reduces the cell death due to induction of NO. The exposure of chondrocytes to the prepared extract before exposure to SNP reduces the cell death significantly ($p<0.05\%$) in dose dependent manner. The increase in viability of cells to approximately must be (50%), 61%, 75% were observed at IM, 50, 100 and 250 µg/mL SXF as shown in Figure 1.



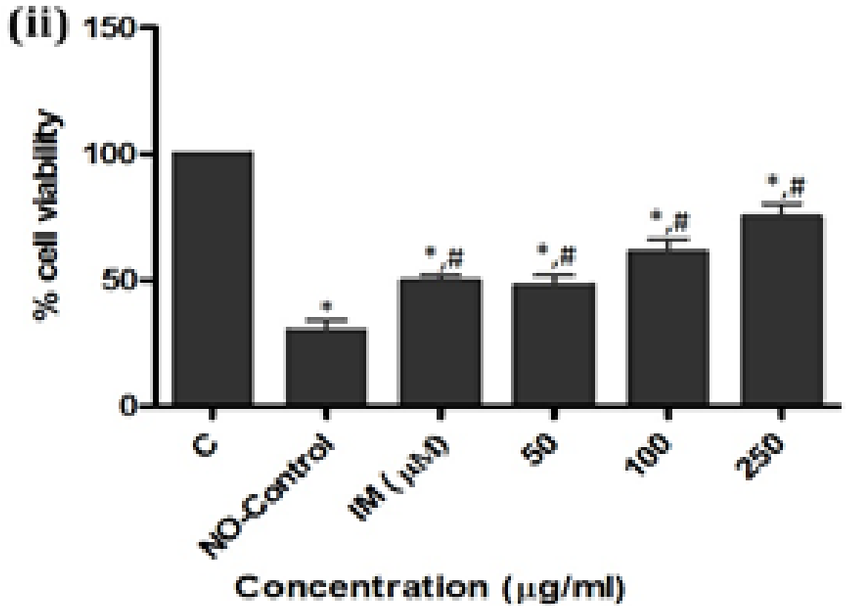
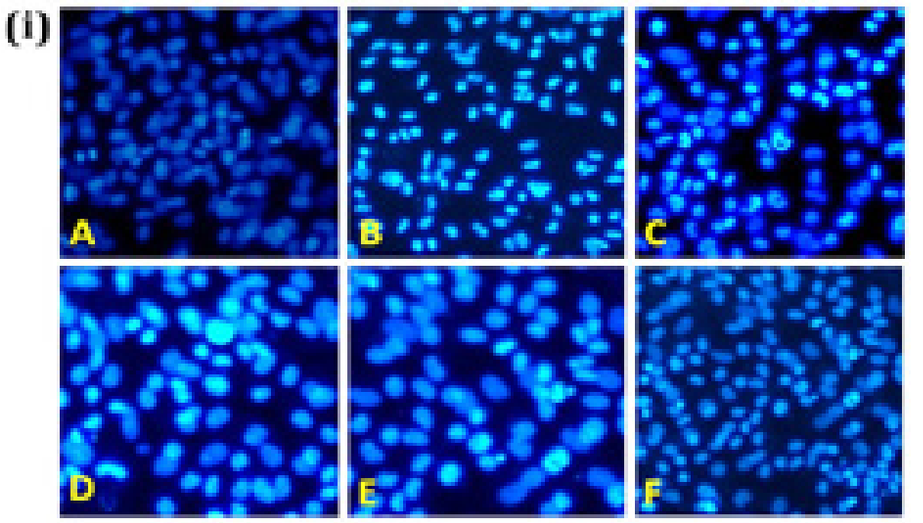


Figure 1. (i) Phase contrast microscopic pictures of chondrocyte at different concentration of SXF extract. (A) Control, (B) NO-Control, (C) IM, (D), (E), (F) at different oncentration of extract (50, 100, 250 μg/mL) (Magnification: 20X; Scale bar: 0.1 mm). (ii) Graph represents the effect of SXF on decreased apoptosis and increased % cell viability in dose dependent manner at different concentrations. Values are obtained from three independent experiments and expressed as mean SEM. *P< 0.001 compared to control and #P< 0.05 compared to NO induced control (NO-Control). (Magnification = 20X and scale bar = 0.1 mm).

3.3 Nuclear Apoptosis Assay

It was observed from photomicrograph (Figure 2), the cells exposed to only SNP (NO-control) shows deep blue fluorescence with condensed nuclei as compared to normal control cells with no fluorescence. The reduction of fluorescence is visualized in IM treated cells and cells treated with 50 μg/mL of SXF compared to NO-control and it further reduces significantly at concentration 100 and 250 μg/mL of SXF.



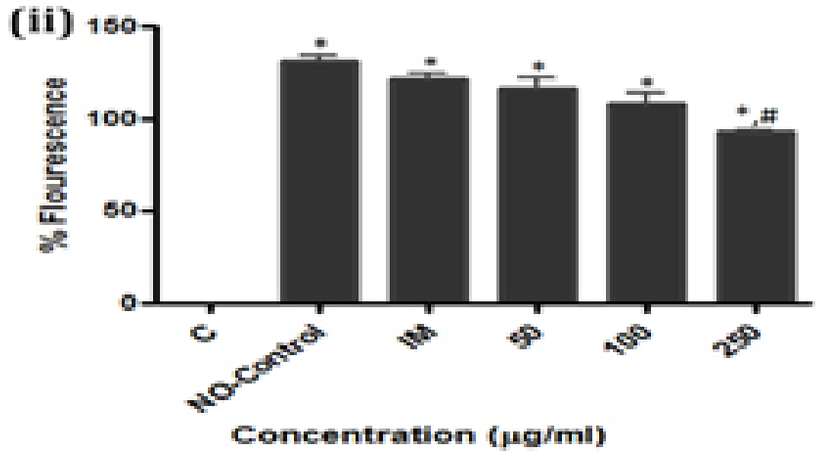


Figure 2. (i) Fluorescence microscopy images showing nuclear condensation in chondrocytes treated with (A) Control, (B) NO-control, (C) IM and (D), (E), (F) different concentrations of SXF (50, 100, 250 µg/mL) (Magnification= 20X and scale bar= 0.1 mm). (ii) Shows graphic representation of % apoptotic cells respective to their controls. The cells were counted manually under a fluorescence microscope in at least 10 random fields and percent apoptotic cells were calculated as detailed under materials and methods. Values were obtained from three independent experiments and expressed as mean and SEM. *P< 0.001 compared with control and #P< 0.05 compared to NO-control.

Furthermore, about approximately 31% condensed cells were observed in SNP-control cells compared to control. The numbers of apoptotic and condensed cell reduced to 21.50%, 16.47%, 8.18% and 2.67% in treated groups IM 25 µM, 50, 100 and 250 µg/mL SXF doses respectively.

3.4 Hoechst-PI Staining

Hoechst-PI double staining showed a decrease in the rate of apoptosis with an increase in the concentration of SXF (Figure 3). The cells with blue and white fluorescence were undergo-

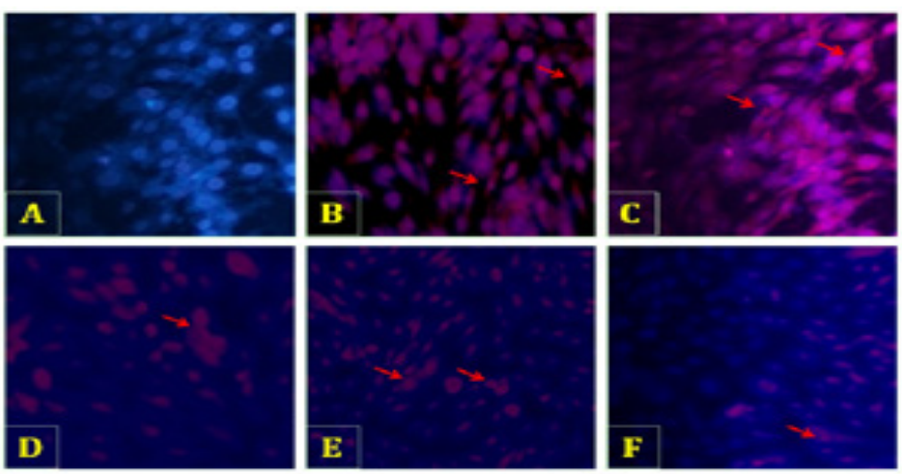


Figure 3. Fluorescence microscopy images showing the cells were treated with SXF extract and 25 µM of IM except control group. Pretreated cells were exposed to SNP for 24 h. Arrow marked the apoptotic cells with pink flouresence. (A) Control, (B) NO induced control without any treatment, (C) IM treated cells, (D), (E), (F) treated with 50, 100, 250 µg/mL of SFX extract. (Magnification: 20X; Scale bar: 0.1 mm).

ing apoptosis and cells with pink fluorescence were dead. The NO-control group without any treatment shows maximum fluorescence. The degree of fluorescence was slightly reduced in IM treated group and 50 µg/mL SXF treated cells. However, the fluorescence was significantly reduced at 100 and 250 µg/mL concentration of SXF.

3.5 Inhibition of ROS Formation

The exposure of cells to hydrogen peroxide (H_2O_2) (10 µM stock solution) for 24 h, significantly reduces the number of chondrocytes. The microscopic examination from fluorescence microscope shows that the intensity of fluorescence was decreased with increase in the concentration of dose of SXF

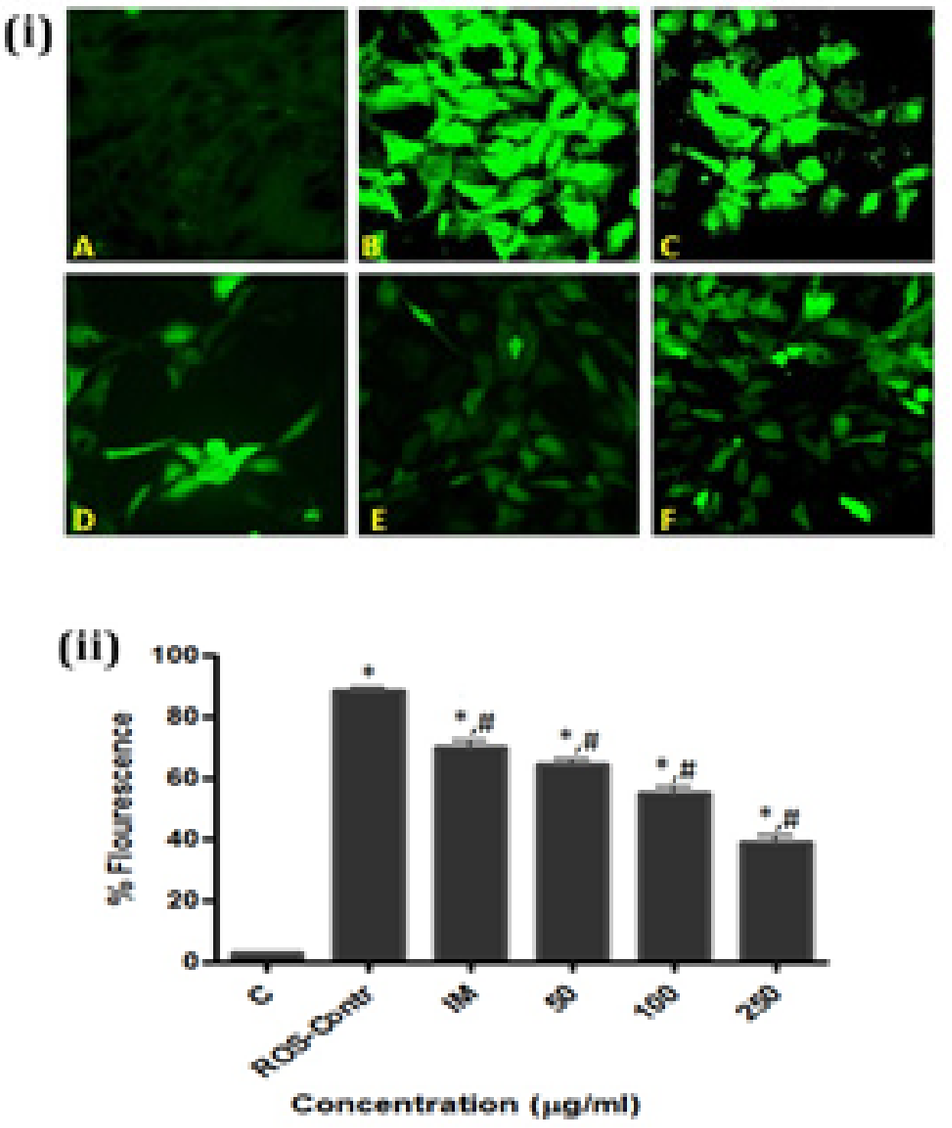


Figure 4. (i) Photomicrographs of chondrocytes showing antioxidant properties of SXF. Cells exposed to H_2O_2 for 24 h before treatment of IM and SXF extract (Magnification: 20X; Scale bar: 0.1mm). (A) Control, (B) H_2O_2 control, (C) IM, (D), (E), (F) at different concentration of extract (50, 100, 250 µg/mL). (ii) Graph representing percentage of ROS generating cells and calculated as DCF positive cells to total number of cells. Data are represented as mean and SEM. Non-parametric test one way ANOVA: *P< 0.001 versus control and #P< 0.05 versus H_2O_2 induced ROS control (ROS-Contr).

i.e., from 50 to 250 µg/mL. The cells, which were exposed to IM shows significant decrease in fluorescence. Quantitative data analysis also demonstrates the significant increase ($p<0.001$) in intracellular ROS production when exposed to 10 µM H_2O_2 is 71.45%. However, when the cells were treated with IM, 50, 100 and 250 µg/mL of SXF, the production of intracellular ROS decreases about 63.41%, 64.63%, 58.70% and 39.10% respectively and thus increases the cell viability as shown in Figure 4.

4. Discussion

OA is a degenerative joint disease with several etiological risk factors.[27]Herbal plants produce safety profile compared to the NSAIDs[28]. From the phytochemical screening of SXF extract shows the presence of various phytochemicals *viz.* flavonoids, anthraquinone glycosides, steroids, alkaloids, terpenoids, tannins and saponins. The phytochemicals detected were known to have certain medicinal importance. Alkaloids derived from plants show an anti-inflammatory property[29]. Phenolic compounds have anti-oxidative, anti-inflammatory, anti-diabetic and anti-carcinogenic properties[30]. Saponin was also known to act as anti-oxidants having anti-inflammatory, weight loss ability and other pharmacological activities[31]. Plant steroids have cardiotonic activity and generally used in herbal medicine and cosmetics[32]. Tannins have astringency property *i.e.*, faster healing up of a wound and mucous membrane[33]. The plant polyphenols have significance, as they are anti-oxidants and free radical scavengers. Polyphenolic compounds have aromatic benzene ring that substitutes hydroxyl radical and its functional derivatives. They can absorb free radicals and can chelate metal ions that catalyze the formation of ROS that promotes lipid peroxidation. Among the polyphenols, flavonoids help to fight against diseases. Its antioxidant potency depends on the molecular structure and position of hydroxyl group including other features in its structure[34].

There is a reduction in the level of oxidative stress produced due to exposure of cells to H_2O_2 pretreated with SXF extract was observed that might be due to the presence of the various anti-oxidant, anti-inflammatory and anti-apoptotic components in the extract. A study supports our finding that antioxidant present in Sumac leaves induced chondrogenesis through preventing ROS generation[35]. In a study, Zhuang *et al.,* 2016 also describes the inhibitory effect of *Angelica sinensis* that protect the chondrocyte from H_2O_2 induced apoptosis in rat chondrocytes through its anti-inflammatory, antioxidant and anti-apoptotic properties[36]. The anti-apoptotic activities on chondrocytes were observed in cells pretreated with SXF extract for 24 h and then exposed to SNP-generated NO-induced cell death. There are many studies that provide the correlation between the level of NO production and chondrocyte apoptosis. The effect of SNP over chondrocytes was evaluated with MTT assay for cell viability, nuclear condensation assay and Hoechst-PI staining for apoptosis of cells. In a study, Lee *et al.,* has demonstrated cilostazol protect the chondrocytes from nitric oxide induce apoptosis[37]. It was observed from statistical data and photomicrography that SXF induces proliferation of cells and reduces the apoptosis of chondrocytes in dose dependent manner. The medicinal properties of SXF are due to the presence of various phytochemicals including various polyphenols like flavonoids. These flavonoids possess anti-oxidant properties due to the indigenous origin and strong tendency to scavenge free radicals[38]. Other antioxidants curcumin and quercetin inhibit inflammatory processes and protect chondrocytes[39]. The antioxidant resveratrol protect chondrocytes apoptosis *via* effects on mitochondrial polarization and ATP production. In a study Beecher *et al.,* 2007 suggested that antioxidant blocks cyclic loading induced chondrocyte death[40]. The data from nuclear condensation suggest that SXF protects chondrocyte from apoptosis. As phytochemicals of SXF contains antioxidants therefore it might be prevent apoptosis.

5. Conclusion

The result demonstrates the presence of certain phyto constituents in the ethanolic fruit extract of *Solanum xanthocarpum* collected from local area of Lucknow, India contains alkaloids, tannins, saponins, flavonoids, steroids and cardiac glycosides. Moreover, the ethanolic extract of SXF shows antioxidant activity by reducing the ROS formation and inhibiting NO-induced cell death in primary chondrocytes.

6. Acknowledgement

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