Current Trends in Biotechnology and Pharmacy
Vol. 16 (4) 445 - 455, Oct 2022, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2022.4.78

Antimelanogenic and Anticancer Activity of Isorhamnetin isolated from *Acalypha indica Linn.* on A375 cell line.

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Abstract:

Hyperpigmentation refers to the skin darkening which is mainly due to the increased production of melanin. Many synthetic drugs are available in the market to cure hyperpigmenta tion but produce various side effects. Medicinal plants are the way forward to curing hyperpig mentation with less side effects and beneficial to the consumers. *Acalypha indica* is one of the traditional medicinal plants that is used for treating various skin disorders but has not been explored for hyperpigmentation and other aesthetic treatments. Therefore, the present study is aimed at isolation of active compounds from *Acalypha indica* for antimelanogenic and anticancer activity on human melanoma cell line A375. The ethyl acetate fraction of *A.in dica* was chosen from the sequential fraction since it has flavonoids and polyphenols in high quantity when compared with other fractions. The compound was isolated using column chro matography technique and further character ized by various analytical techniques including mass spectra, FT-IR spectra, and ^{13}C-NMR and ^{1}H-NMR. Cytotoxicity study on A375 melanoma cell line was studied using MTT assay and the IC_{50} value was calculated. The mode of cell death was studied by acridine orange and ethid ium bromide staining. Melanin and Tyrosinase activity was studied using the cell line. Western blotting was performed using various pro-apop totic and apoptotic genes. Results revealed that the compound isolated was Isorhamnetin and the compound showed significant antiproliferative activity with the IC_{50} value of 8.26 µg/ml. The cell cycle analysis revealed that the com pound arrested the cells at sub G0/G1 phase with the depletion of cells in S and G2-M phase. The anti-apoptotic genes such as Caspase 3, Caspase 9 and BCL-2 were down regulated and Bax protein was upregulated.

Keywords: A375 cell line, Isorhamnetin, Cell cycle arrest, Docking, Bax Protein

Introduction

In humans, skin pigmentation diverges among various populations based on the level of UV penetration. Population near the poles and located far from the tropical region receives very low intensity of UVB radiation. Contrastingly, the populations near the equator region are exposed to high amounts of UVB radiation. Exposure to UVB radiation induces the process named as melanogenesis by the organelle called melanosomes (1). Dysregulation of melanisation may lead to hyperpigmentation or hypopigmentation. Among the different dermatological disorders, variation in pigmentation is the third common disorder which causes high psychosocial impairment (2). Various skin disorders including skin melanoma, freckles, nevus,

and age spots are mainly due to hyperpigmen tation due to increased production of melanin pigment (3). Therefore, hyperpigmentation can be treated by the regulation of melanisation pro cess (4).

The age-old drug for treating this hyperpigmentation is hydroquinone which is a hydroxyl phenolic compound. Because of the adverse effects of this drug like contact derma titis, skin irritation etc., hydroquinone has been removed from the cosmetic industry in various countries including the European Union (5). An other drug used in the cosmetic industry as a skin bleaching agent is tretinoin. These drugs are the synthetic agent involved in the inhibition of the tyrosinase activity. Due to the various side effects of these synthetic compounds, identifi cation of compounds from natural sources with little or no side effects are the need of the hour (6). In the present study, the plant *Acalypha in dica* was chosen because of its traditional use in skin disease including skin eruptions (7). Pre liminary studies in our laboratory showed that the ethyl acetate extract had potent anti-mela nogenic activity on zebrafish embryos and sig nificant cytotoxicity on A375 melanoma cell line. Therefore the present study is focused on iso lating an anti-melanogenic compound from the ethyl acetate fraction of the *Acalypha indica* and identifying the mechanism of action.

Materials and Methods

Maintenance of cell line

A375 melanoma cell line was obtained from National Centre for Cell Science, Pune, India (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100µg/ml) in a 5% CO_2 at 37° C.

Isolation of active compound

The ethyl acetate extract was dis-solved in methanol and adsorbed on silica gel 60 – 120. After evaporation of the solvent it was loaded into a silica gel column (100 – 200 mesh size), prepared in hexane. The column was elut ed with hexane followed by gradually increas ing polarity with Hexane: ethyl acetate (90:10; 80:20; 70:30; 60:40; 50:50; 40:60; 30:70; 20:80) and finally with 100% Ethyl acetate. The column was further eluted with Ethyl acetate: Chloro form (90:10; 80:20; 70:30; 60:40; 50:50; 40:60; 30:70; 20:80) and finally with 100% Chloroform. The column was further eluted with Chloro form: Methanol (98:2; 94:2; 92:8; 90:10; 85:15; 80:20). A total of 124 fractions were collected and monitored under TLC. The fractions 43 to 56 showed similar bands and these fractions were combined and the solvent was evaporat ed under reduced pressure. The resulting crude materials were purified by using activated char coal in hot ethanol and the fractions are kept for crystallization. The obtained crystals were ana lyzed further for mass spectra, FT-IR spectra, and ^{13}C-NMR and ^{1}H-NMR (8).

MTT assay using isolated compound

The anticancer activity of the isolated compound was studied in the A375 cell line. $5*10^3$ cells were seeded in the 96 well plate and incubated at 37 °C for 24 hours. Once the cell reaches 80% of confluency, media was removed. The cells were treated with different concentrations of the isolated compound and incubated again for 24 hours. After the incuba tion period, the test drug was removed and 100 µl of the MTT prepared in PBS was added and further incubated for 4 hours. After the incuba tion period was complete, the formazan crystal formed was dissolved using DMSO and the ab sorbance was measured at 570 nm (9).

Estimation of melanin content in cell line

The melanin content was estimated by a slightly modified method (10). A375 cells were cultured at a density of 10^5 cells per well in 96 well microtiter plates at 37 °C overnight. The test sample (0.31, 6.25, 1.25, 2.5, 5 and 10 µg/ ml) was added to well, and incubated for 24 hr. Then, the supernatant was removed and wells

were rinsed with phosphate buffer saline (PBS) twice. The detached cells were transferred to 1.5 ml micro-tubes and centrifuged at 1100 rpm for 7 min. 100 µl of 2 M NaOH was added to cells, incubated at 100 °C for 30 min and optical density was measured using a microtiter plate reader at 405 nm.

Estimation of tyrosinase activity

A375 melanoma cells were cultured in 96 well microtiter plates. The cells were mixed with different concentrations (0.31,0.62,1.25, 2.5,5 and 10µg) of the extract and incubated for 24 hr. The supernatant was removed and the cells were washed with PBS twice. The detached cells were transferred to 1.5 ml micro-tubes and centrifuged at 1100 rpm for 7 min. To the pellet, 100 µl of lysis buffer containing 100 mM Na_3PO_4 (pH=6.8) was added to the cells and incubated for 30 min in ice cold condition. After centrifugation at 10000 rpm at 4 °C for 20 min, 100 µl of 5 mM L-dopa was added to 100 µl of supernatant (each containing 100 µg of the protein) at 37 °C for 2 hr. Tyrosinase inhibition was calculated by measuring the absorbance of each well and comparing it with the control group (without plant extract) at 475 nm (10).

Acridine orange and ethidium bromide staining:

To visualize the apoptotic body formation and nuclear changes after the drug treatment, the cells were stained with AO/EtBr. Briefly $5x10^4$ cells were seeded in the six well plate and incubated at 37 °C for 24 hours for reaching the confluence. Once the cells reached the 80% confluency, the cells were treated with different concentrations of the isolated compound for 48 hours. After the incubation period, the media was removed and the cells were fixed with methanol: glacial acetic acid (3:1). After fixing the cells, the plate was washed with PBS and stained with a 1:1 ratio of AO/EtBr. Once the cells were stained, it was observed under fluorescence microscopy with a magnification of 40X (9)

DNA fragmentation

The A375 melanoma cell line was seeded in the 6 well plate at the density of $5x10^3$ cells. After treatment, the cells were harvested by pipetting and rinsed with ice-cold buffer. The cell pellets were resuspended in 200 µl of lysis buffer consisting of 0.2% Triton X-100, 10 mM Tris, and 1 mM EDTA (pH 8.0). After incubation for 10 minutes, the samples were centrifuged at 5000g for 5 min. The DNA in the supernatant was extracted using a 25:24:1 (v/v/v) equal volume of phenol: chloroform: isoamyl alcohol. The DNA was precipitated with ethanol, air dried and dissolved in the TE buffer (pH 8.0). The samples were analyzed electrophoretically on 1% agarose gel containing 0.1 µg/ml ethidium bromide. The entire procedure was repeated for the control Vero cell line (11).

Western blotting

The cultured A375 cells were treated with different concentrations of drugs for 48 h. After 48 hours of treatment, the media was removed and washed with PBS. The cells were washed with a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 100 µg/ml PMSF, 1 µg/ml Aprorinin, 1% NP-40). After lysis, the cells were centrifuged and the supernatant was taken in a separate tube. The proteins were separated using 10% SDS-Polyacrylamide Gel and the gel was transferred to the PVDF membrane. The membrane was incubated with the primary antibody for the different proteins including p53, Bax, BCl2, Caspase 3 and Caspase 9 (12).

Cell cycle analysis by fluorescence-activated cell sorting (FACS)

To check the inhibitory effect on cell cycle, FACS was performed. After treatment, control and treated cells were washed with ice-cold phosphate-buffer saline (PBS). The cells were scraped using rubber policeman and centrifuged at 2000 rpm for 10 min. Then, cells were mixed with 500 µl of hypotonic solution (1% sodium citrate, 0.04% RNAse, 0.05% Propidium

Iodide (PI), and 3%Triton X-100) and the distri bution of cells in different phase was determined by C6 Accuri (BD) flow cytometer (9).

Docking

The 3D structure of the 5M8M.pdb was downloaded from the protein data bank. Molecular docking was performed using the Autodock4.2 software. The 3D structure of mol ecules was optimized using chemdraw 13.0 using MMFF 94 (Maximum number of interac tion: 5000, minimum RMS gradient: 0.100). All avoidable water and ligand were removed from the enzyme and the default docking parameters were fixed and docking was performed.

Results and Discussion

Structural elucidation of compound

The molecular weight of the isolated compound was determined by high-resolution mass spectrometry and the molecular ion peak appeared at m/z 317.09 (M-1). The ^{1}H NMR spectrum of compound revealed several reso-nances in the aromatic region 7.67 – 7.69 (1H, d, Ar-H); 7.75 (1H, s, Ar-H); and 6.19 (1H, s, Ar-H); 6.47 (1H, s, Ar-H); 6.94 – 6.93 (1H, d, Ar-H). The doublet peaks with the magnitude of the coupling constants were observed indicating meta coupling. Three typical broad hydroxyl sig nals were 9.44 (1H, s, -OH); 9.74 (1H, s, -OH); 10.76 (1H, s, -OH); and a sharp signal appeared at 12.46 (1H, s, -OH) indicating an alcoholic pro ton chelated by a ketone. Peak at δ 55.73.74, indicated the presence of methoxy moiety which was correlated with 1H-NMR signal at 3.84. The proton signals appeared at δ 6.19 – 7.75 were correlating with carbons at δ 175 – 102.99 re spectively.

The ^{13}C-NMR spectrum showed signals at δC 175, 148.78, and 135.79 ppm typical of C-4, C-2 and C-3, respectively, which confirms the flavanone structure. The chemical shifts at δC 175, 163, 160, 135 ppm were corresponding to three aromatic carbons connected to hydrox yl groups at C-5, C-7 and C-3, C-4' respective ly. The flavonoid ring junctions appearing at δC 98.16 and 155 ppm correspond for carbons C-9 and C-10 respectively. The carbonyl suggested by the 1H-NMR spectrum was confirmed by the ^{13}C-NMR resonance observed at δC 175.84.

The FTIR spectra showed the peaks at 3289 cm^{-1} - 3402.78 (-OH group), 3138 (=CH stretch), 3101 (–CH3 asymmetric stretch). The carbonyl group C=O stretching band was lo cated at 2360 and aromatic double bond 2340 cm-1. In the fingerprint region, the –CH bend ing vibration peaks were observed at 1505 and 1162 cm^{-1}.

This pattern of the spectrum was identi cal with that of compound Isorhamnetin [1]. The structure of compound-1 as shown in Fig-1.

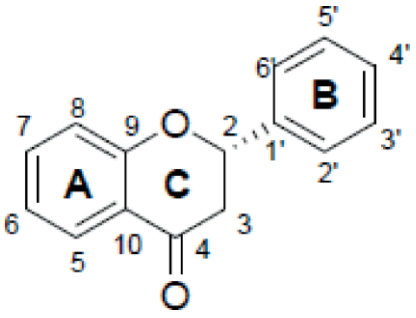


Fig 1: Flavonoid ring system

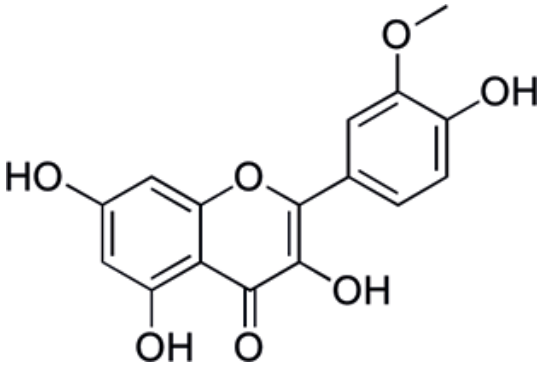
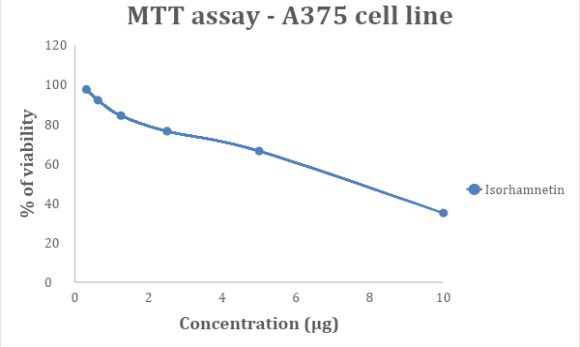


Fig 2. Isorhamnetin

The cytotoxicity analysis of the isolated com pound was studied using MTT assay on A375 melanoma cell line. The dose dependent de crease in the cell viability was observed after 24 hours of incubation. The IC_{50} value for the isolated compound was found to be 8.26 µg.



Graph 1: Cytotoxic effects of isolated compound against A375 melanoma cells. Cell viability was calculated and dose dependent decrease in cell viability was observed in A375 cells. The mechanism of cell death was evaluated through apoptosis or necrosis.

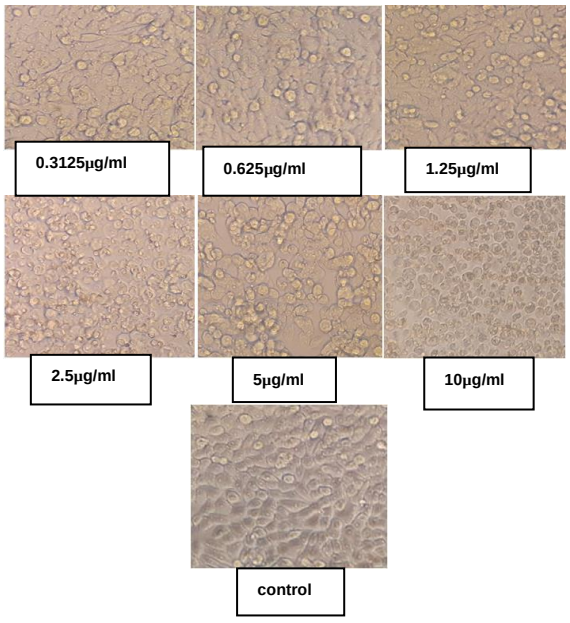


Fig 3: Morphological changes observed in A375 cell line. Phase contrast images revealed the distorted morphology in the treated cell line.

AO/EtBr staining:

The mechanism of cell death was evaluated through apoptosis or necrosis. The cells were stained with AO/EtBr and observed under fluorescent microscopy. In the control group, the intact cells with green fluorescence were observed. In the compound treated cells, early apoptotic, late apoptotic, necrotic cells were observed (Fig 4).

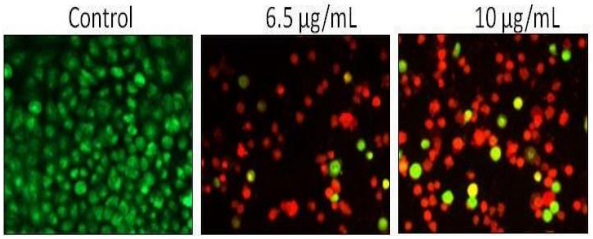


Fig 4: Control cells showed live cells whereas treated cells showed apoptotic and necrotic population dose dependently.

DNA fragmentation

DNA fragmentation reveals DNA dam age in the A375 melanoma cells treated with Isorhamnetin. In the control Vero cells, intact DNA was observed. Cells treated with the com pound at concentration of 6.5 µg/ml and 10 µg/ml showed fragmented DNA which initiates the apoptotic mechanism in the cells (Fig 5).

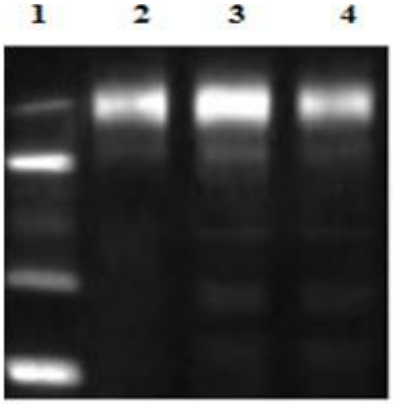


Fig 5: Control cells showed intact DNA whereas treated cells showed fragmented DNA in treated cells. Lane 1) Marker (1000 kb); 2) Control; 3) Cells treated 6.5 µg/ml of Isorhamnetin; 4) Cells treated 10 µg/ml of Isorhamnetin.

Cell cycle analysis

Distribution of cells in various phases of cell cycle was examined using flow cytometry. Results showed accumulation of cells in sub G0/G1 phase in dose dependent manner when compared to control (Fig 6). The depletion of cells in S and G2-M phase was also observed in the treated cells.

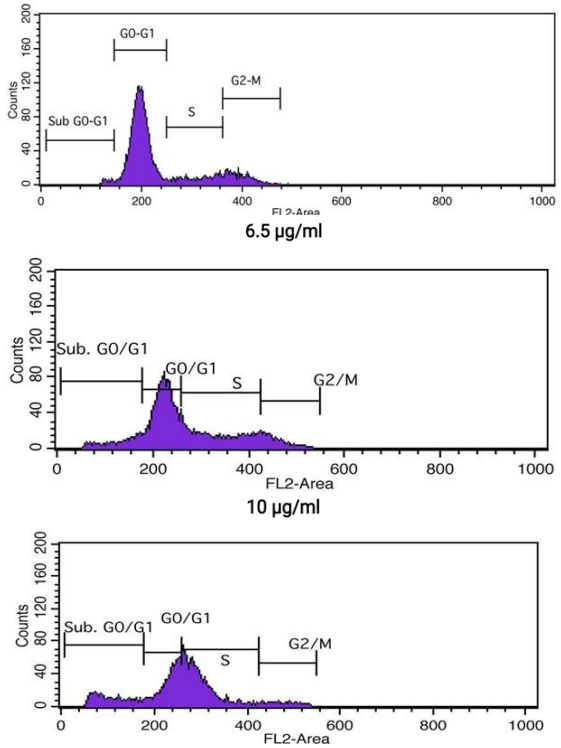


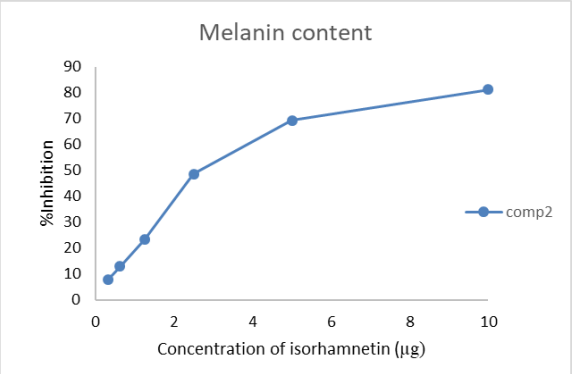
Fig 6: Cell cycle analysis by flow cytometry

Estimation of melanin content

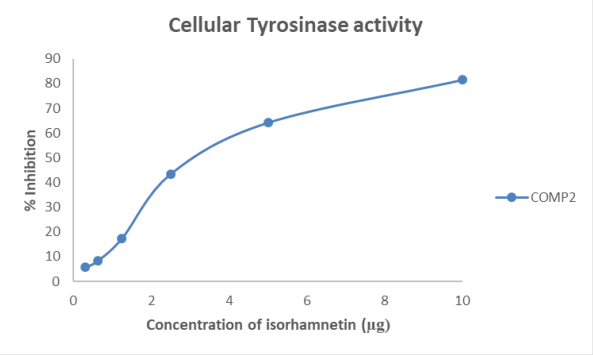
To study the effect of the isolated com pound against hyperpigmentation, melanin content in the A375 melanoma cell line was es timated. At 100 µg/ml concentration of the com pound, the inhibition of melanin content was found to be 80%. The IC_{50} value for the melanin inhibition assay was found to be 2.6 µg/ml.

Estimation of tyrosinase activity

The inhibition of hyperpigmentation ac tivity was further confirmed by the cellular tyrosi nase activity. Percentage of tyrosinase inhibition was calculated and inhibition of the enzyme increased in dose dependent manner.



Graph 2: Inhibition of Melanin content of the isolated compound in A375 cells.



Graph 3: Inhibition of tyrosinase activity of the isolated compound in A375 cells.

Isorhamnetin upregulates the BAX protein

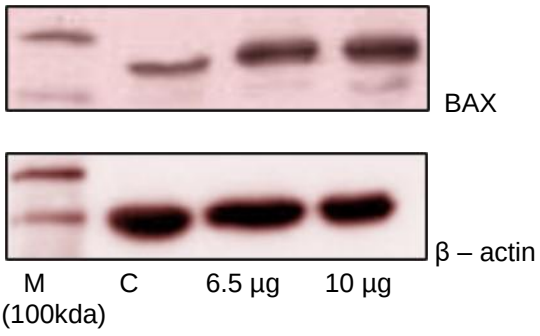


Fig:7 Western blot image of Bax protein. Bax was upregulated in Isorhamnetin treated cells while it was downregulated in control cells.

Anticancer activity of Isorhamnetin was further confirmed by the analysis of expression of various proapoptotic and apoptotic markers like BAX. Results showed increased expression of BAX in the compound treated groups when compared to control.

Role of Isorhamnetin in inhibition of BCL-2 protein

BCL-2 plays a vital role in apoptotic processes therefore the role of Isorhamnetin in inhibition of BCL-2 was studied. BCL-2 expression was upregulated in the control cells where as in the treated cells there was marked reduction (Fig 8).

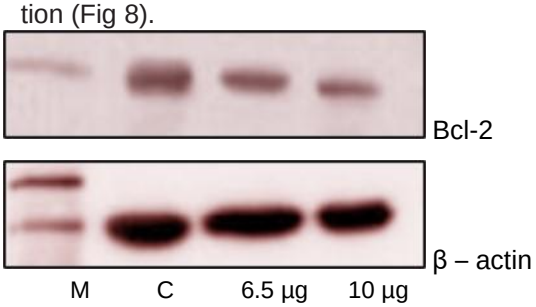


Fig:8 Western blot image of BCL2 protein. BCL2 was upregulated in the control cells with concomitant decrease in the Isorhamnetin treated cells.

Expression of caspase 3 and caspase 9 in A375 cell line:

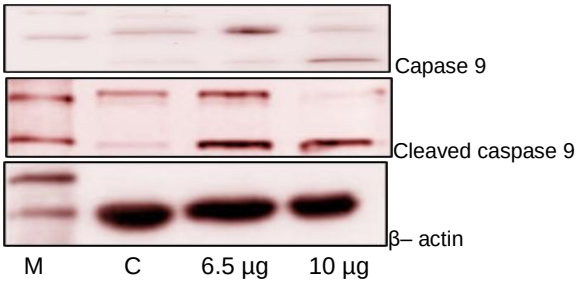


Fig:9 Western blot image of caspase protein. Caspase 3 and 9 was upregulated in the control cells with concomitant decrease in the Isorhamnetin treated cells. Cleaved caspase 3 and caspase 9 was down regulated in the control cells whereas increased expression was observed in Isorhamnetin treated cells.

Role of Isorhamnetin in the expression of caspase 3 and caspase 9 was studied using western blot. Both the caspases 3 and 9 and its cleaved form was expressed in the Isorhamnetin treated cell line when compared with the control. Caspase 3 and 9 expression was inhibited by the Isorhamnetin in the A375 cell line (Fig 9).

Upregulation of P53 expression in Isorhamnetin treated cell line

Western blot analysis of p53 expression reveals 10 µg Isorhamnetin upregulates the expression of p53 when compared with the control (Fig 10).

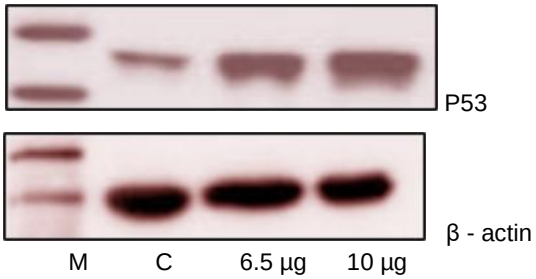


Fig 10: Western blot image of p53. p53 was downregulated in the control cells and the expression of p53 in the Isorhamnetin treated cells was upregulated.

Molecular docking of 5M8M with Isorhamnetin

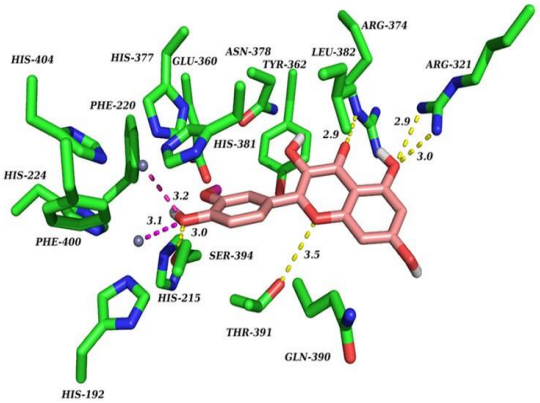


Fig 11: Interaction of Isorhamnetin with Human Tyrosinase (5M8M)

Table 1: Interaction of ligands Isorhamnetin (3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl) chromen-4-one) with 5M8M.pdb

Compound ID	Binding Energy (Kcal/mol)	Inhibition constant (µM)	Interacted aminoacid	Bonding Distance Å	Bonding Types	Binding site of protein (5M8M)	Binding site of Ligand
Isorhamnetin	-5.64	73.07	SER394	3.0	Conventional hydrogen bonding	Oxygen of SER394	Oxygen of 4-hydroxy of phenyl
			ARG321	2.9	Conventional hydrogen bonding	Nitrogen of ARG321	Oxygen of 5-hydroxy of chromenone
				3.0		Nitrogen of ARG321	Oxygen of chromen-4-one
			ARG374	2.9	Conventional hydrogen bonding	Nitrogen of ARG374	Oxygen of chromen-4-one
			THR391	3.5	Conventional hydrogen bonding	Oxygen of THR391	Oxygen of 4-hydroxy of phenyl
			CU2+	3.2	Conventional hydrogen bonding		Oxygen of 3 methoxy phenyl
			CU2+	3.1	Ionic interactions		
					Ionic interactions		

Isorhamnetin showed high binding capacity with the 5M8M protein with the binding energy of -5.64 Kcal/mol. The compound effectively binds with the following amino acids *viz* SER394, ARG321, ARG374, THR391 with the bonding distance of 3.0 Å, 2.9 Å, 2.9 Å and 3.5 Å respectively (Table 1).

In the present study, Isorhamnetin; the compound isolated from *Acalypha indica, was* examined for anticancer activity in A375 melanoma cell lines. A375 is the human malignant melanoma cell line that has the characteristic feature of cutaneous melanoma with the mutation of B-RAF and CDKN2 (13).

Cell viability of A375 cells were examined using MTT assay which showed decreased cell viability dose dependently with an IC_{50} value of 8.26 µg/ml for the isolated compound, Isorhamnetin from *A.indica* ethyl acetate fraction. The isolated compound did not show any cell death in the Vero cell line up to 100 µg/ml. Since the compound did not show any cytotoxicity in the normal cell line; further studies were carried out to investigate the anticancer activity of Isorhamnetin. Once the cytotoxicity is observed, the cells will undergo apoptosis or necrosis (14). In order to check the rate of cells undergoing apoptosis or necrosis, the cells were stained with AO/EtBr. The compound induced

both apoptosis and necrosis in a dose depen dent manner. To further investigate the role of Isorhamnetin exhibiting anticancer properties, the mechanism of apoptosis should be studied. Apoptosis can be initiated and the result can be evaluated by morphological changes, elevation of biochemical markers, chromatin condensa tion and fragmentation in the nucleus and DNA damage (15). DNA damage in the cell line was observed using DNA fragmentation assay and the result showed dose dependent fragmenta tion of DNA in Isorhamnetin treated cells when compared to control.

Cell cycle is the common pathway oc curring in the cell; which comprises various checkpoints for cell division. Escaping from the cell cycle is one of the common strategies for the cancer cells to undergo rapid divisions (16). Duration of cell cycle in both the normal and tu mor cell will be the same but the proportion of cell division is high in cancer cells when com pared to normal cells (17). In the present study, the cell cycle inhibition was observed at G0/ G1 phase and depletion of cells in the subse quent phases. In previous studies, Isorhamne tin treated in A549 lung cancer cell line showed G2/M phase arrest (16). To further confirm the anticancer activity, the level of protein BCLl-2, Bax and p53 was estimated using western blot technique. BCL2 and BAX are the important proteins in apoptosis where the former protein is involved in inhibition of apoptosis, while the latter is involved in promoting the apoptotic mechanism. p53 is a tumor suppressor protein that has an important role in cell cycle progres sion and induces apoptosis (18). In this study, expression of BAX and p53 in the control group was reduced but it was increased dose-de pendently in the Isorhamnetin treated groups. Once the apoptotic signal is received, the ini tiator caspase 9 will get activated and caspase 3 will be involved in the execution of apoptosis (19). In this study, the caspase 3 and caspase 9 were upregulated in the Isorhamnetin treated groups when compared to the control. Molecular Docking results revealed high binding efficacy of Isorhamnetin with 5M8M Human tyrosinase protein (Fig 11). The type of interaction and the affinity of the compound to bind between the target protein and the ligand can be analyzed by the hydrogen bond formation and binding energy. Stable and stronger bonds are indicated by the lower binding energy since the affinity of the ligand to bind the target protein is higher. In this study, the melanogenesis enzyme target protein tyrosinase-related protein 1 (TRP 1) (PDB ID: 5M8M) was chosen to study the inhibitory activity of the compound to melanogenesis enzyme. The lower binding energy value between the compound and the TRP1 indicates stronger interaction between the compound and melanogenesis enzyme, hence isorhamnetin has the potential to inhibit melanogenesis enzyme and depigmentation. The binding energy of Isorhamnetin was found to be -5.64 Kcal/mol; which is lower, therefore, stronger and stable bonds with higher affinity are produced between the ligand and the target protein. The bond distance value of less than or equal to 3 Å indicates that the method is valid and the compound can be further be taken for docking analysis (20). The compound isorhamnetin was found to be equal to 3 Å which showed a good and valid root mean square distance value. This showed that the compound Isorhamnetin has a potential to exhibit anti melanogenic and depigmentation activity to tyrosinase-related protein 1 (TRP 1) (PDB ID: 5M8M).

Conclusion

The present study showed the anti-cancer and anti melanogenesis activity of Isorhamnetin isolated from *A.indica* ethyl acetate fraction on A375 cell line. The compound inhibited the melanin content and tyrosinase activity. Similarly the compound showed anticancer activity by downregulating the anti-apoptotic genes and upregulating the proapoptotic genes.

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