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Antioxidant activity of Isolated and semi synthesized 5-methoxy-2-(3, 4-dimethoxyphenyl)-4-oxochroman-7-yl 4-substitutd benzoate derivatives from *Citrus aurantium*

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Abstract
The present study evaluated the antioxidant activity of the methanolic extract, isolated hesperidin and hesperitin and a hesperitin derivative semi synthesized from *Citrus aurantium.* Extracting using soxhlet apparatus to obtain methanolic extract from the orange peel of Citrus aurantium followed by isolation of a vital flavonoids glycoside (hesperidin and hesperitin), and synthesizing a new hesperitin derivative. The antioxidant activity was, evaluated by 1,1- diphenyl-2-picryl hydroxyls, (DPPH) free radical scavenging activity methods. The antioxidant potential was, determined by IC_{50} Value. The isolated hesperitin Aa showed higher biological activity and semi synthesized derivative (Aa-03) showed potent antioxidant activity as compared to other derivatives.

Keywords: *Citrus aurantium*, hesperidin, hesperitin, antioxidant activity

Introduction
Orange peel is the fresh or dried outer portion of the Rutaceae family. *citrus aurantium* pericarp, among the most important citrus fruits in the world [1-2]. It is generally cultivated in the tropical and subtropical regions of the world, and many other regions, with a total production of about 102 million tons, due to its beautiful colours, pleasant flavors; citrus fruits are well received by consumers from all around the world. Citrus fruits are highly widely consumed as healthy food, juice, and the peel is most frequently discarded as waste containing a wide range of secondary components with considerable antioxidant activity relative to other parts of the fruit [3-5].

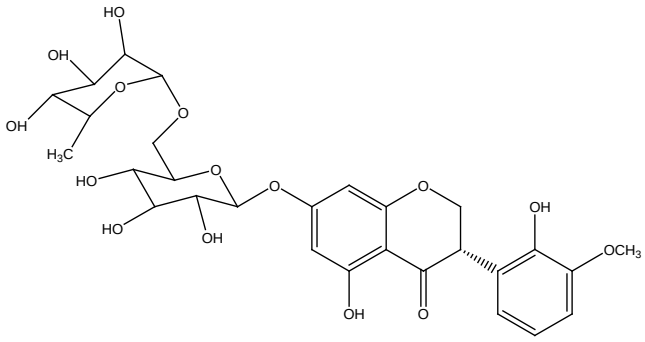
The bitter orange (Citrus aurantium) varies in some characters, but it is similar to the orange. Fruits are an important source of compounds called phytonutrients such as ascorbic acid, flavonoids, carotenoids, phenolic acid, tocopherol and sulphur containing compounds, which possess antioxidant properties. Citrus fruits belong to the family Rutaceae, are one of the main trees grown throughout the world. Citrus fruits are richer sources of bioactive compounds having beneficial effect on human health such as Vitamin C, carotenoids, flavonoids, limonoids, essential oils, alkaloids and minerals.[8-9] Phytochemicals are non-nutritious plant chemicals with defensive or preventative properties for diseases. Most of the Phytochemicals have antioxidant activity and protect our cells against oxidative damage and reduce the risk of developing certain types of cancer. Bitter orange peel contains limonene (about 90%) volatile oil, flavonoids, triterpenes, monoterpene, vitamin C, carotene, and citric acid. The peel of the immature fruit is used for indigestion, stomach pain, constipation and dysenteric vomiting, and diarrhea in traditional Chinese medicine [12-15].

Hesperidin is a polyphenolic bioflavonoid form of flavanones, found abundantly in the skin and membraneous parts of the orange peel and other citrus fruits. The natural product found in the citrus peel for the food industry and human wellbeing, sugars, flavonoids, volatile oils is relative of use. Orange peels can also be used as an antioxidant and a good source of plant-derived beneficial compounds. Leaves, fruit, bark, flower, and root are historically used in the treatment of diseases. It is also called on sour orange, Bigarade orange, Seville orange, Citrus aurantium (Khatta: Hindi, Narangam Tamil) which is grown in India and its fruits and used for various medicinal purposes.[10-11] The special medicinal benefits of bitter orange have been acknowledged despite its efficiently admirable importance as a source of edible fruits [18].

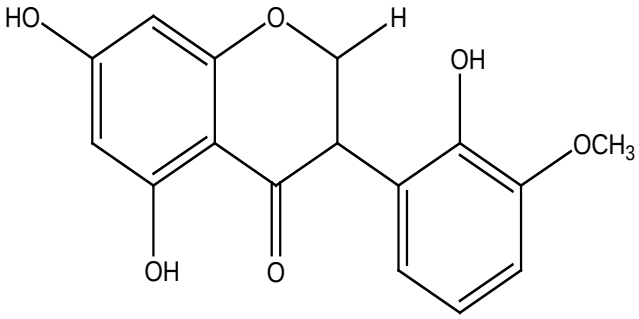
Fig 1: Bitter orange fruit and fresh orange peel, dried orange peel& peel powder

Hesperidin: Importance and use
Hesperidin is hesperitin-7-beta-rutinoside; its aglycone is 4'-methoxy-3', 5,7-trihydroxy flavanones; and its sugar moiety rutinose is linked to the C-7 position of the aglycone. It is the predominant flavonoids of lemon and sweet orange. Hesperidin is a glycoside of flavanone composed of aglycone, hesperetin, and rutinose, an attached disaccharide.

The disaccharide unit is composed of one molecule of rhamnose and one of glucose [20]. It is the predominant flavonoid in citrus species, occurring mainly in the peel and membranous parts of or In addition, it has many biological effects including decreasing capillary fragility, anti-inflammatory, antimicrobial, antioxidant, and anti-carcinogenic effects.[22]

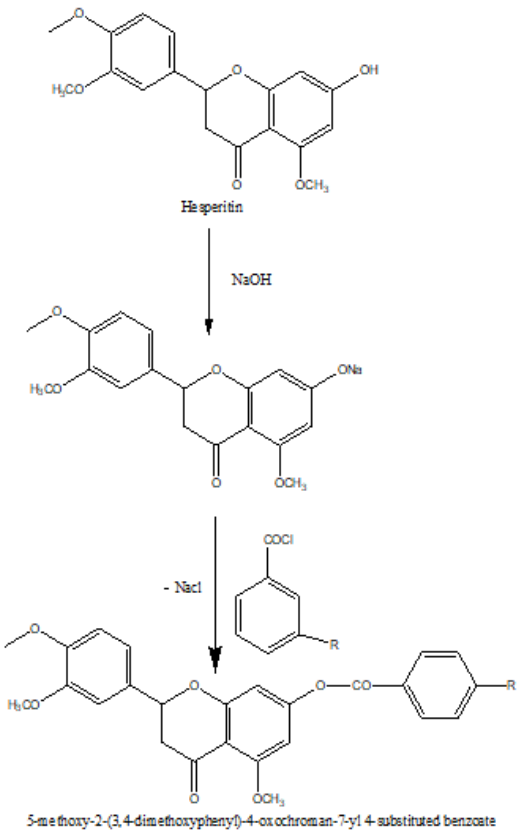


Hesperidine



Hesperitin

2. Scheme



Types of substituted benzoate

Table 1

Compound	R
R1	-OCH_3
R2	-Cl
R3	-OH

Material and method

1. Collection of plant material

The peel of *citrus aurantium* used for the present studies were collected from local market of Lucknow India. The plant was Identified, Confirmed and Authenticated by CSIR- Central Institute of Medicinal and Aromatic Plants (Council of scientific and Industrial Research) Lucknow-226015, India. (Reference No:CIMAP/Bot-Pharm/2020/07)

2. Extraction of Crude Hesperidin

Air-dried orange peel was extracted in Soxhlet assembly with different solvents in a series of solvent extractions like n-hexane, petroleum ether (40-60℃), chloroform, 95% ethanol and water. The average extractive values of different extracts were calculated [21].

2.1 Procedure: Extraction of hesperidin from *Citrus aurantium*

100 gm powder of *Citrus aurantium* peel with 400ml of petroleum-ether (40-60℃) On water both and reflux for one hrs. Filter the contents while hot through a Buchner funnel and dry the marc at room temperature.

The powder extract with 400 ml of methanol under reflux for 3hrs. filter while the hot and wash the marc with 50 ml of, hot methanol. Add to the washing to the filtrate.

Concentration the combined filtrate under reduced pressure to syrupy mass extract was kept in desiccators to remove the excessive moisture and was used for further isolation.

Fig 2: Extraction by Soxhlet Apparatus

2.2 Isolation of Phytoconsituents from the *Citrus aurantium*

Orange peel is dried & 50g of the fined powder is subjected to reflux with 200 ml of alcohol for 1.5 to 3 hrs. After the crude hesperidin was added to 7 ml of dimethylformamide then added acetic acid solution at 60 ^{0}C. The solution was filtered through a Buchner funnel and dilute with water

allow to stand for 4hrs the Deabsorbed & extracted in to chloroform. White crystals of Hesperidin are separated out.

2.3 Conversion of hesperidin into hesperitin

Hesperidin, methanol, and concentrated sulfuric acid were stirred together and heated for 8 hours at reflux. After cooling and concentration, the homogeneous solution is diluted with ethyl acetate. The organic solution is washed and dried with magnesium suphate after being washed with water. Purification of hesperitin was achieved by dissolving the crude product in a small amount of acetone and then adding the standard solution to a vigorously stirred solution of water and acetic acid. Water is used to wash and cool precipitated hesperitin in an ice bath. Hesperitin is purified into a pure yellow powder. [7]

Recrystallization

When separated from extraction, the solid organic compounds are rarely pure. They are often contaminated with other compounds (impurities) generated in the same process as the desired product. Purification of impure crystalline compounds is normally accomplished by using a suitable solvent or solvent mixture in crystalline form. The variations in solubility in a given solvent or mixtures of solvents are used to purify solids by crystallization.

1. Dissolving the impure matter of boiling point or in suitable solvent.
2. Filtration to precipitate the solution from insoluble materials & dust particles.
3. Allowing a reaction mixture to cool until the dissolved material crystallizes.
4. Isolating the crystals from the solution by removing them from the solution and bringing them to the surface. Make use of technology or mother-liquor. On drying, the resulting solid is checked for purity using a melting point determination, a spectroscopic process, or TLC, and if found impure, it is recrystallized using fresh solvent. The procedure was repeated until the compound was completely pure.

2.4 Procedure for synthesis hesperitin derivatives

Hesperidin, methanol, and concentrated sulfuric acid were stirred together and heated for 8 hours at reflux. After cooling and concentration, the homogeneous solution is diluted with ethyl acetate.

The organic solution is washed and dried with magnesium suphate after being washed with water. Purification of hesperitin was achieved by dissolving the crude product in a small amount of acetone and then adding the standard solution to a vigorously stirred solution of water and acetic acid. Water is used to wash and cool precipitated hesperitin in an ice bath. Hesperitin is purified into a pure yellow powder.

After reaction of hesperitin. Further hesperitin react was react with sodium hydroxide

And reflux for 2hrs. and after completed reaction to form intermediate compound. Further intermediate react with benzyl chloride and to form substituted derivative.

Hesperitin (0.01 mol) is dissolved in diethyl ether and refluxed with various phenolic (0.01mol) acids to produce ester derivatives of (A1-01) (S)- 5- methoxy -2-(3,4-dimethoxyphenyl)-4- oxochroman-7-yl 4- hydroxybenzoate, (A2-02) (S)- 5- methoxy -2-(3,4-dimethoxyphenyl)-4-oxochroman-7-yl 4- methoxybenzoate, A3-03) (S)- 5-methoxy -2-(3,4-dimethoxyphenyl)-4- oxochroman-7-yl 4-chlorobenzoate, newly synthesized hesperitin derivatives.

Identification of an isolated compound

Isolated compounds from the extracts of *citrus aurantium* were identified by using the following technique.

1. Determination of melting point range

The melting points of the compounds were calculated using a melting point apparatus and an open capillary process. Compounds were mounted in a sealed capillary on one end and caves built for the capillary on the other. In the caves, a thermometer was set up. Melting point range refers to the temperature range between when a compound begins to melt and when it finally melts.

1.1 Method development for thin layer chromatography (TLC)

TLC was performed on glass plates coated with silica gel G. Using a traditional spreader, the adsorbent silica gel G was coated to a thickness of about 0.3 mm on previously cleaned TLC plates of 20 x 5 cm. For activation, the plates were put in a hot air oven for 30 minutes at 110°C. Around 2 cm above the lowest part of the powered plate, the compounds were added as a spot. The polarity of the products n was used to select the mobile phases. -Butanol: Butanol is a type of alcohol that is used to the mobile step was butanol: acetic acid: water (3:1:1). When the mobile process had completed 3/4 of the plate, sports were imaged using a UV chamber (340nm) Rf value was determined.

2. Solubility studies

Solvents such as water, toluene, methanol, ethanol, chloroform, acetone, n-hexane, and petroleum ether were used to dissolve the 10mg extracted products in a 50 ml beaker. For the isolated compound, measurements were taken.

2.1 Description of solubility

Table 2

S.No.	Descriptive terms	Pergram of solvent, the approximate amount of the solvent in millilitres
1	Very soluble	Less than 1
2	Freely soluble	From 1 to 10
3	Soluble	From 10 to 30
4	Sparingly soluble	From 30 to 100
5	Slightly soluble	From 100 to 1000
6	Very slightly soluble	From 1000 to 10000
7	Insoluble or practically insoluble	More than 10000

3. Spectral analysis

3.1 UV spectral analysis

10 mg of compounds is dissolved in appropriate solvents and diluted to 20 ml in the same solvent. The above solution was diluted to 50 mL from 2.0 mL. A UV-Visible Spectrophotometer was used to collect UV spectra.

3.2 IR spectral analyses

IR of compounds using potassium bromide molecules in the perkina Elmer Spectrum RXI FTIR system. Spectrums were record.

Preparation of KBr pellet of compounds

100 mg of anhydrous KBr (AR grade) was measured precisely, and 1.0 mg of the compound was applied and triturated thoroughly. The mixture was put in an evacuated die and pressed for 5 minutes at a pressure of 5-6 tonnes. The IR spectra were captured using a transparent disc that was then put in a pellet holder.

3.3 H NMR spectral analysis

TMS was used as an internal standard to record 1H NMR spectra of compounds on a Bruker Avance-300 NMR. (chemical shift δ in ppm).

4. Biological activity of the isolated compound

4.1 Antioxidant Activity

The purple colored methanol solution of DPPH was used to determine the hydrogen atom or electron donation potential of the corresponding extracts. The stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is used as a reagent in this spectrophotometric assay. A effective result with a concentration range of 20, 40, 100, 200 and 400 µg/mL was added to one ml of 0.1 mM methanol solution of DPPH in methanol. After 30 minutes in the dark, the absorbance of the solution mixture is estimated at 517 nm. A blank, 1 mL of DPPH solution was used with UV-VIS spectrophotometer (UV-1800, Shimadzu Corp., Japan).

4.2 Pharmacological studies

4.3 Antioxidant activities assay

4.4 Radical scavenging activity on 1, 1-diphenyl-2-picryl Free hydrazyl (DPPH)

Preparation of 0.004% w/w solution of DPPH

4 mg of DPPH dissolve in 40 ml methanol & volume make up to the 100 ml volumetric flask then sample were incubated at dark place in 30 min. Prepared 0.004 % solution of DPPH.

Preparation of stand. ascorbic acid solution

10 mg Ascorbic acid in 5 ml methanol and volume make up to the 10 ml volumetric flask. Prepared 1 mg/ml solution of ascorbic acid.

Firstly take 5 test tube and were taken to make aliquots of 5 concentrations (20, 40, 100, 200, and 400 µl/ml), this conc. ascorbic acid takes into 3 ml of DPPH solution in a test tube.

Further, repeat procedure isolated compound in the test tube and 3 ml DPPH added in a test tube and incubated at a dark place in 30 min and absorbance was taken from the 517 nm with UV-Visible spectrophotometer. Observed the absorbance of standard and tested solution, then note observed value.

DPPH scavenge activity (%) Inhibition = Abs – Abc /Abs ×100

Where,

Abs: Absorbance of blank sample

Abc: Absorbance of the sample

Result and discussion

Table 3: Physical Character of hesperidin and hesperitin

S. No	Parameter	Observation Hesperidin	Observation Hesperitin (A1)
1.	**Color**	Yellowish brown	Yellowish
2.	Odor	Characteristic and Aromatic	Characteristic and Aromatic
3.	Melting point range	242-244 ^{0}C	226 ^{0}C
5.	TLC:-n-butanol: Acetic acid: Water (4:1:5)	0.61	0.72

Table 4: Morphology of three isolated compounds

S.No	Compound Code	Colour	Odour	Taste
1.	Aa-01	Yellowish Colour	Aromatic	Bitter or pungent
2.	Aa-02	Yellowish Colour	Aromatic	Bitter or pungent
3.	Aa-03	Yellowish Colour	Aromatic	Bitter or pungent

Table 5: Physiochemical characters of Hesperitin Derivatives

S.No.	Compound Code	Molecular Weight	Molecular Formula	TLC R_f value	Melting point (ºC)	Percentage Yield	Colour
1.	Aa-01	450.13	$C_{25}H_{22}O_8$	0.57	219	65	Yellowish
2.	Aa-02	468.88	$C_{25}H_{21}ClO_7$	0.61	225	67	Yellowish
3.	Aa-03	464.15	$C_{26}H_{24}O_8$	0.72	227	70	Yellowish

(S)-5-methoxy-2-(3,4-dimethoxyphenyl)-4-oxochroman-7-yl 4-hydroxybenzoate (Aa-01)

Solubility data: Acetone, methanol, ethanol, ethylacetate, acetonitril, water

Spectral data:

FTIR (KBr, cm-1): 3509 cm-1 (O-H stre), 3055cm-1 (C-H stre (arene), 3600 (Are-OH Stre), 2947cm-1 (C-H stre, alkane), 2839 CH stre.(alkane-OCH_3), 1642 (C=O stre), 1612 (Ar-C=C stre), 1492 (C-C.(Aro), 1198 (-O- ether), 950 (C-C stre. Ali), 750 C-Cl stre).

1H NMR (DMSO-d6) δ (ppm): 1H NMR (DMSO-d6) δ (ppm): -5.51-6.59 (m 4H, Ar-CH), 3.13- 3.38 (s, 2H CH_2), 6.30 (s, 2H), 3.73 (s, 3H,CH_3), 6.88-7.97 (m, 4H, Ar-CH), 7.97 (s, 1H, CH), 5.0 (Ar-OH), 3.73(6H, CH_3).

(*S*)-5-methoxy-2-(3,4-dimethoxyphenyl)-4-oxochroman-7-yl 4-chlorobenzoate (Aa-02)

Solubility data: Acetone, methanol, ethanol, ethylacetate, acetonitril, water

Spectral data:

FTIR (KBr, cm-1): 35091cm-1 (O-H stre), 3055cm-1 (C-H stre (arene), 2947cm-1 (C-H stre, alkane), 2839 CH stre.(alkane-OCH_3), 1642 (C=O stre), 1612 (Ar-C=C stre), 1492 (C-C.(Aro), 1198 (-O- ether), 950 (C-C stre. Ali), 750 C-Cl stre).

1H NMR (DMSO-d6) δ (ppm): 5.51-6.59 (m 4H, Ar-CH), 3.13- 3.38 (s, 2H CH_2), 6.30 (s, 2H), 3.73 (s, 3H,CH_3),

7.42-8.08 (m, 4H, Ar-CH), 3.73 (s, 6H, CH_3). 3.37(s, 3H, CH3), 3.36 (s, 3H, CH3), 3.30 (s, 3H, CH3)

(S)-5-methoxy-2-(3,4-dimethoxyphenyl)-4-oxochroman-7-yl 4-methoxybenzoate (Aa-03)

Solubility data
Freely soluble**:** Water, acetic acid, ethanol, methanol, ethyl acetate

Spectral data
FTIR (KBr, cm-1): 35091cm-1 (O-H stre), 3055cm-1 (C-H stre (arene), 2947cm-1 (C-H stre, alkane), 2839 CH stre.(alkane-OCH_3), 1642 (C=O stre), 1612 (Ar-C=C stre), 1492 (C-C.(Aro), 1231(-OCH_3 stre), 1198 (-O- ether), 950 (C-C stre. Ali).

1H NMR : 1H NMR (DMSO-d6) δ (ppm): -5.51-6.59 (m 4H, Ar-CH), 3.13- 3.38 (s, 2H CH_2), 6.30 (s, 2H), 3.73 (s, 3H,CH_3), 3.73-8.03 (m, 5H, Ar-CH), 3.73 (s, 9H, CH_3)

Antioxidant activity
Showed the inhibitory concentration of isolated compound (A1-01) (S)- 5- methoxy -2-(3,4-dimethoxyphenyl)-4-oxochroman-7-yl 4- hydroxybenzoate required to scavenge 50% DPPH free radicals (IC50 in µg/mL). Though the entire isolated compound exhibited free radical scavenging ability. IC50 value for isolated (A1-01) (S)- 5- methoxy -2-(3,4-dimethoxyphenyl)-4- oxochroman-7-yl 4- hydroxybenzoate compound was found to be very low (2.15 to 35.34 µg/ml) this indicated their high ability to scavenge DPPH free radicals. Among isolated compound of hesperitin showed lowest radical scavenging activity. The ascorbic acid standard compound also showed high ability to scavenge DPPH. Ascorbic acid and the isolated compound were showed comparatively low DPPH radical scavenging ability with high IC50 values. Significant variation was observed among varieties and also among species in DPPH radical scavenging ability.

Evaluation of antioxidant activity
Results for antioxidant activity of the hesperitin test from the ascorbic acid standard by DPPH method.

DPPH.Scavenging activity (%) Inhibition = [(Abs – Abc)/Abs]×100

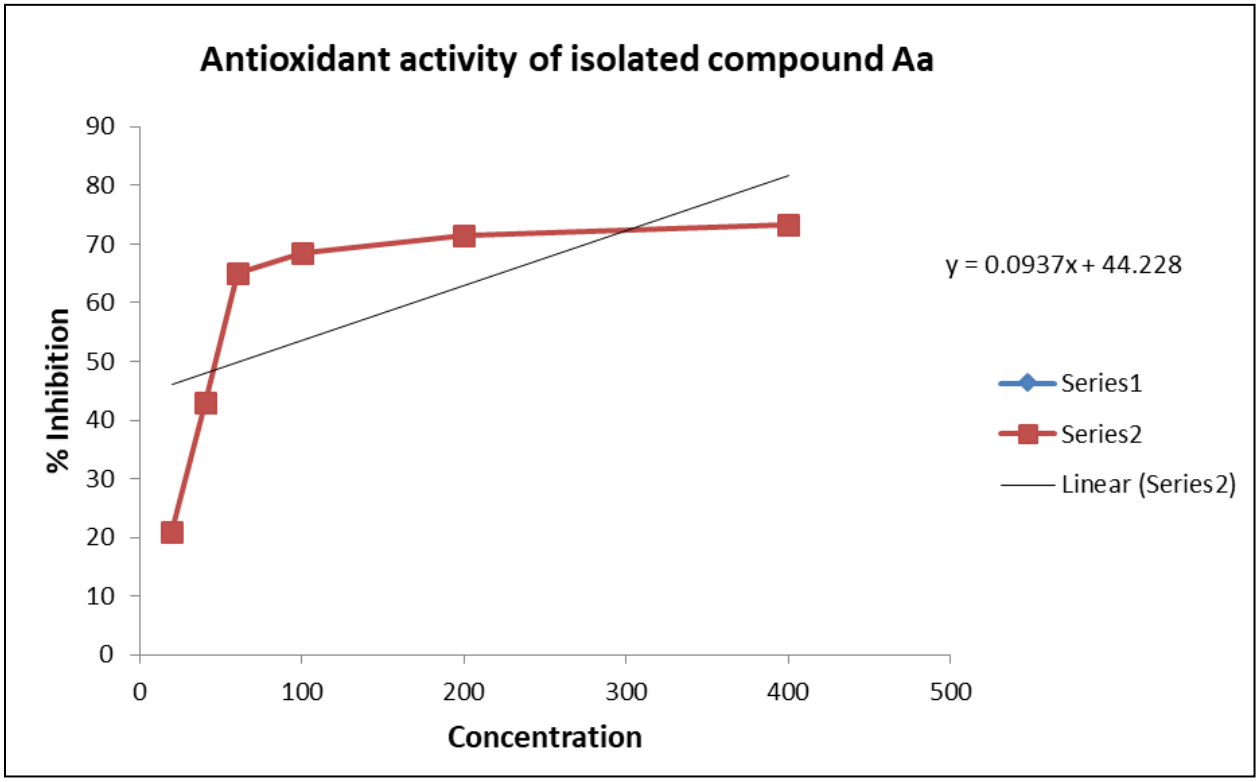
Where, Abs = absorbance of blank sample
Abc = absorbance of sample

Table 6: Compound- Aa

S.No.	Concentration µg /ml	Absorbance (std)	% Inhibition
1	20	0.278	72.52
2	40	0.210	80.4
3	60	0.115	88.63
4	100	0.017	98.32
5	200	0.010	99.0
6	400	0.008	99.02

Table 7: percentage Inhibition antioxidant activity of Ascorbic acid (Stan dard)

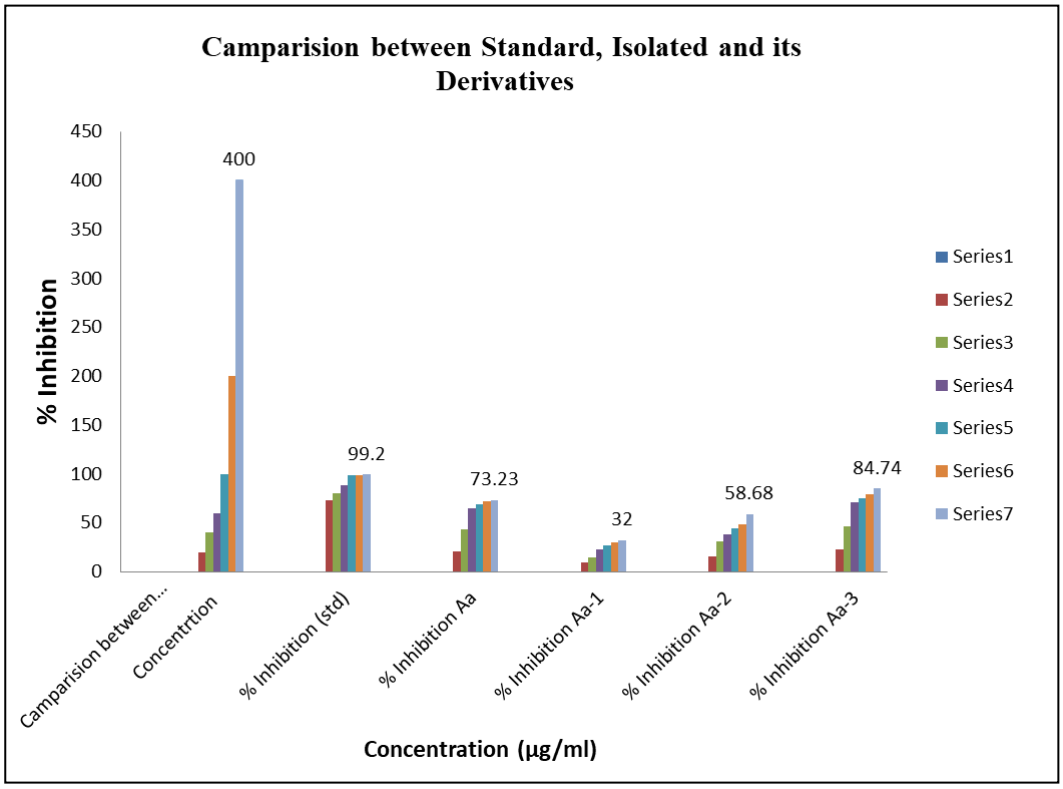
S. No.	Concentration µg /ml	Absorbance (std)	% Inhibition	IC50 (µg/ml)
1	20	0.871	20.96	
2	40	0.627	43.10	
3	60	0.385	65.06	65.15
4	100	0.348	68.42	
5	200	0.315	71.41	
6	400	0.295	73.23	



Graph 1: The graph shows the derivative (Aa) compound increased antioxidant activity with increasing conc.

Table 3: Comparison between antioxidant activity of standard, isolated compound and its derivatives

Concentration (µg/ml)	20	40	60	100	200	400
% Inhibition (std)	72.52	80.4	88.63	98.32	99	99.2
% Inhibition Aa	20.96	43.1	65.06	68.42	71.41	73.23
% Inhibition Aa-1	9.06	14.22	22.31	26.66	30.22	32
% Inhibition Aa-2	15.65	30.79	38.32	44.14	48.14	58.68
% Inhibition Aa-3	22.25	46.33	70.98	75.1	79.3	84.74



Graph 2: The graph shows that high activity in ascorbic acid (Std) then isolated compound (Aa), and its derivatives (Aa-01, Aa-02, Aa-03)

Conclusion

The present study evaluated the antioxidant activity of the methanolic extract, isolated hesperidin and hesperitin and a hesperitin derivative semi synthesized from *Citrus aurantium*. Extracting using soxhlet apparatus to obtain methanolic extract from the orange peel of Citrus aurantium followed by isolation of a vital flavonoids glycoside (hesperidin and hesperitin), were extracted and isolated from orange peel citrus aurantium and characterized by thin layer chromatography TLC and melting point, chemical test mentioned in the results. The antioxidant potential was, determined by IC_{50} Value. The isolated hesperitin (Aa) showed more potent antioxidant activity as compared to derivatives.

Showed the inhibitory concentration of isolated compound (A1-01), (A1-02), (A1-03), required to scavenge 50% DPPH free radicals (IC50 in µg/mL). Though the entire isolated compound exhibited free radical scavenging ability. IC50 value for isolated (A1-02, A1-03) compound was found to be (15.65 to 58.68 µg/ml),(22.25 to 84.74) this indicated their good ability to scavenge DPPH free radicals. Among isolated compound of hesperitin showed lowest radical scavenging activity. The ascorbic acid standard compound also showed high ability to scavenge DPPH. Ascorbic acid and the isolated compound were showed comparatively low DPPH radical scavenging ability with high IC50 values. Significant variation was observed among varieties and also among hesperitin species in DPPH radical scavenging ability.The isolated and synthesizing a new hesperitin derivative. The antioxidant activity was, evaluated by 1,1- diphenyl-2-picryl hydroxyls, (DPPH) free radical scavenging activity methods. The antioxidant potential was, determined by IC_{50} Value. The isolated hesperitin (Aa) and semi synthesized derivative, (Aa-01), (Aa-02) less potent antioxidant activity as compared to derivative (Aa-03). the isolated hesperitin derivative Aa-03) showed potent antioxidant activity as compared to other derivatives.

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