CHEMICAL AND BIOLOGICAL SCREENING OF *NIGELLA SATIVA* LINN. GROWN IN STATE OF JAMMU & KASHMIR

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خلاصہ

نائی جیلا ستائی وا کے بیج بہت ارصہ پہلے سے ایشا، مشروسطہ اور دیگر ممالک میں مختلف علاج میں استعمال ہوتا ہے۔ نائی جیلا کے بیجوں کا امیزہ اینٹی ریڈیکل، اینٹی بیکٹریل، اینٹی ٹیومر اور سوزش کے خلاف فعال ہے۔ موجودہ تحقیق میں اس پائے جانے والے اجزا کا پتا لگایا گیا ہے۔ اور اس میں الکلائیڈ، سزائڈ فلوینائیڈ، ٹرپینائیڈ، فی نول، کیمرین اور کاربوھائیڈریٹ دیکھے گئے ہیں۔ جبکہ فلوبوٹینن اور اموڈول موجود نہیں ہیں۔ اینٹی بیکٹریل کا عمل چار بیکٹریا کے اقسام کے خلاف کیا گیا جبکہ اینٹی اکسیڈینٹ پر بھی کام کیا گیا ہے۔

Abstract

Nigella sativa Linn. (Kolonji) seeds are extensively used for medicinal purposes in Asia, Middle East and Far East countries from a long time. Extract of *Nigella sativa* seeds have ability to show anti-radical, anti-bacterial, anti-tumor and anti-inflammatory activities. Present study was carried out to detect some important compounds which are responsible for these activities. Through phytochemical screening it was revealed that alkaloids, steroids, flavonoids, terpenoids, phenols, coumarine and carbohydrates are present in methanolic extract of *Nigella sativa* seeds while pholabotannins and emodol are absent. Anti-bacterial activity was performed against *S. aureus, E. coli, B. subtilis, B. pumilus, P. aeruginosa* bacterial strains and maximum zone of inhibition was observed against *E. coli* and *P. aeruginosa.* Methanolic extract of *Nigella sativa* seeds were also screened for anti-radical scavenging activity by using DPPH and ABTS methods. Total phenolic contents (TPC) and total flavonoid contents (TFC) were determined in quantitative screening.

Introduction

Nigella sativa is a local annual, dicotyledonous herb of *Ranunculaceae* family. It is also known as fennel flower plant. Maximum height of this plant is about 60cm.There are different names of *Nigella sativa* in different languages in different regions of the world e.g. Kalonji (Urdu), Habba-tl-sawda (Arabic), black cumin (English), Shonaiz (Persian), Kalajira (Bangali) (Khan, 1999). *Nigella sativa* grows in different countries e.g. Pakistan, India and Middle East countries. It is also indigenous growing plant in Arab countries and (Jansen, 1981). The seeds of *Nigella sativa* are known as black cumin seed and they are very important in many pharmacological studies for its immune modulatory and therapeutic properties (Salem, 2005). About 100 compounds are present in black cumin seeds. The most important compounds due to which medicinal value of these seeds increased are saponins, flavonoids, volatile oil and trace elements (Salih *et al.*, 2009).

Seeds of *Nigella sativa* are being used for thousands of years as folk remedies for number of traditional diseases (Toncer and Kizil, 2004). Egyptians, Greeks considered the medicinal importance of this plant from a long time. In Egypt from a long time oil of *Nigella sativa* has been used for severe cough and asthma (Zawahry, 1963). It is observed that many pharmacological activities such as antioxidant, anticancer, anti-inflammatory, antineoplastic and anti-asthmatic activities are shown by this miraculous medicinal plant (Saheb *et al.*, 2016).

Nigella sativa in traditional medicines as well as in recent years has been used for the treatment of microbial diseases without any reported side effect. It has been considered worldwide as an important medicinal herb. Its use in pharmaceutical, food and ornamental industries displays considerable value. Enough literature is available about the seeds of *Nigella sativa* but in this study we tried qualitatively and quantitatively investigate the phytochemicals in the whole plant which can be responsible of the pharmacological uses.

Material and Methods

Sample preparation

Seeds of *Nigella sativa* were collected from District Bhimber (AJ&K) and pulverized by using house-hold electric grinder. Solvent extraction was performed to extract oil from seeds. Initially seeds of *Nigella sativa*

were placed in shade for 2 to 3 days at 30°C to 35°C temperature to remove all moisture. After that seeds were finally powdered by using electric grinder.

Solvent Extraction

About 250g pulverized seeds were taken and immersed into 500 mL methanol for 10-15 days. Sample was shaken on daily basis to remove unwanted gases and for perfect mixing of sample with solvent. After that sample was filtered by using Buckner funnel under high vacuum through Whatman's filter paper number 1. The process was repeated by soaking residue into methanol for three to four times. The filtrate obtained after filtration was condensed by using high vacuum rotary evaporation. Oil like extract was obtained after complete drying of rotary extract at room temperature. At the end anhydrous sodium sulphate (Na_2SO_4) was added in sample to remove moisture.

Chemicals and reagents

Mayer's reagent**,**Wagner's reagent, Hager's reagent, Chloroform($CHCl_3$), Ferric chloride ($FeCl_3$), Conc.H_2SO_4, Methanol (MeOH), Sodium hydroxide (NaOH), Lead acetate solution, Copper acetate, Dil.HCl, Sodium bicarbonate ($NaHCO_3$),NH_3 Solution, Ethanolic *α*-naphthol, Gallic acid,2,2'-diphenyl-1-picrylhydrazyl(DPPH),2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid(ABTS) and Rutin.

Phytochemical Analysis:

Different chemical tests were carried out for identification of different phytochemical constituents found in methanol extract of *Nigella sativa* seeds. Only authentic and standard methods were used for phytochemical analysis.

Detection of Alkaloids

For detection of Alkaloids following methods were used. Extract by dissolving was prepared 50g seeds in 200 mL of dil.HCl and filtered. The filtrate was tested by using following procedures.

a) Mayer's test: The filtrate was mixed with few drops of Mayer's reagent (potassium mercuric iodide). Formation of white or creamy precipitate indicated the presence of alkaloids.

b) Wagner's Test: In the above filtrate few drops of Wagner's reagent (Iodine in Potassium Iodide) were mixed. Appearance of reddish brown precipitate confirms the presence of Alkaloids.

c) Hager's Test: To the filtrate few drops of Hager's reagent (saturated solution of Picric acid) were added. Prominent yellow color precipitate shows the presence of alkaloids (Tiwari *et al.,* 2011).

Detection of Terpenoids

The test for detection of terpenoids was performed by protocol proposed by Edeoga *et al.* (2005)**.** This method is also known as Salkowski's method. By following this protocol 2.5mL of methanolic extract was mixed with 1mL of chloroform ($CHCl_3$) and then about 1.5mL of conc.H_2SO_4 was added in above mixture. Formation of red coloration specifies the presence of terpenoids.

Detection of Phenols

Few drops of ferric chloride ($FeCl_3$) were added in 3mL of extract. Appearance of bluish black color shows the presence of phenols (Tiwari *et al.,* 2011).

Detection of Steroids

Detection of steroids was carried out following protocol proposed of Edeoga *et al.,* (2005) According to technique presence of steroid is confirm by changing the color shade from violet to green or blue. By using the method 0.5g of sample of *Nigella sativa* was taken then adds ethanol in it. After that 2mL of acetic acid was mixed in it and was stirred with glass rod. After mixing, 2mL of conc. H_2SO_4 was added and shake gradually.

Detection of Flavonoids

a) Alkaline reagent test: Few drops of 10% solution of NaOH were added into extract. Intense yellow color was observed. By addition of dil. HCl color was disappear. This indicates the presence of flavonoids.

b) Lead Acetate test: Few drops of lead acetate solution were added in extract of *Nigella sativa*. Formation of yellow color precipitate indicates the vicinity of flavonoids (Tiwari *et al.,* 2011).

Detection of Saponins

A plant sample (powdered seeds) about 1g was boiled with 10mL of water for few minutes and then filtered. 5mL of filtrate was then mixed with 2mL of distilled water and shake vigorously till the formation of froth. Froth formation confirms the presence of saponins.

Detection of Diterpenes

Copper Acetate test: According to technique vicinity of diterpenes is confirmed by appearance of green coloration. By using this method 0.1g of copper acetate dissolved in 3mL of aqueous filtrate of sample and then mixed vigorously (Tiwari *et al.,* 2011).

Detection of Phlobatannins

This test is used to determine the presence of phlobatannins in the extract. According to protocol of Tiwari *et al.* (2011), 5mL of aqueous extract of *Nigella sativa* was taken in test tube. Then 5mL of 1% HCl was mixed in it. The mixture was boiled for 5 minutes. Formation of red precipitate designates the presence of phlobatannins.

Detection of Carboxylic acid

1mL of extract of *Nigella sativa* was taken. Few mL of sodium bicarbonate ($NaHCO_3$) solution was added in extract. Effervescence showed vicinity of carboxylic acid (Kumar *et al.,* (2013).

Detection of Coumarine

1mL of aqueous extract of *Nigella sativa* was taken in test tube. Test tube was covered with filter paper and few drops of NaOH solution was added in it. For several minutes' test tube was placed in water bath. After sometime filter paper was erased and test tube was passed through UV light. Yellow coloration specified the vicinity of coumarine (Eglinton *et al.,* 1962).

Detection of Emodol

Plant extract was taken 4mL and then concentrated to 2mL. After concentration 1-2mL of 25% ammonia (NH_3) solution was added with continuous shaking. Appearance of cherish-red color designates the presence of emodol in plant extract under study (Nasir and Irshad, 2015).

Detection of Carbohydrates

Molish's test: Aqueous filtrate (2mL) of extract was taken and 0.2 ml of ethanolic *a*-naphthol (20%) was mixed in it. Then 2mL of sulphuric acid (98%) was added carefully from the side of the test tube for the formation of two layers. At the junction of two layers appearance of violet region shows the presence of carbohydrates (Eglinton *et al.,* 1962).

Biological Analysis

Biological analysis involves the study of anti-bacterial activity and anti-oxidant scavenging of *Nigella sativa* seeds extract.

Sample Preparation

About 100g of black cumin seeds were taken and dipped in 200mL of methanol for 10 days. After this period solution was filtered by using Whatman's filter paper Number 1. From sample solution was removed by using rotary evaporator. Methanolic extract of *Nigella sativa* was obtained. In order to remove any moisture from the extract small amount of anhydrous sodium sulphate (Na_2SO_4) was added. Sample was ready for anti-bacterial activity.

Media Preparation

In order to prepare Mueller Hinton Agar media 38g of powdered agar was dissolved in 1 liter distilled water. In order to homogenize the mixture, flask was shaken continuously. Medium was sterilized in Autoclave for 15 minutes at 121^{o}C temperature.

Culture Bacteria

In order to examine anti-bacterial activity of *Nigella sativa* extract *Staphylococcus aureus, Escherichia coli , Bacillus subtilis , Bacillus pumilus* and *Pseudomonas aeruginosa*
bacteria were used.

Agar Well Diffusion Method

A petri plate was taken in which Luria Broth Agar was poured. Agar was solidified after sometime. After solidification 100μL was poured in nutrient agar plate and spread on it by utilizing hygienic spreader. Later on, by using sterilized borer well was made in the middle of nutrient agar plate. The well was filled with 40μL of extract of black cumin seeds. It was left in incubator at 37^{o}C for one day.

In another agar plate, well was made which was filled with 40μL of solvent as a negative control. It was also placed in incubator for one day at 37^{o}C. After one day zone of inhibition was measured in mm by using scale (Nasir and Irshad, 2015).

Free radical scavenging effect of methanolic extract of *Nigella sativa* Linn.

Free radical scavenging activity by using DPPH* (2, 2'-diphenyl-1-picrylhydrazyl)

An effortless, fragile and fast method to determine free radical scavenging activity is by using DPPH* that is spectrophotometrically a stable radical. For determining antioxidant activity of *Nigella sativa* seeds extract, stable 2, 2-diphenyl-1-picryl hydrazyl radical was used (Koleva *et* al., 2002). In this method, methanolic solution of DPPH* radical is reduced into stable DPPH-H by accepting proton from antioxidant (Sallappan and Akoh, 2002). Different concentrations of sample were prepared by dissolving 25, 50, 75, 100μL of extract in DMSO so that total volume of solution will be 1000μL. 1mL of methanolic DPPH solution was added in extract and kept in dark for 30 min. Then absorbance was observed at 517nm (Koleva *et al.,* 2002).

$$DPPH^{\cdot} + AH \longrightarrow DPPH\text{-}H + A^{\cdot}$$

By addition of sample into DPPH solution, absorbance of DPPH solution decreases. Antioxidant activity (% inhibition) is calculated by using formula given below

$$\% \text{ inhibition} = [(Ac - As)/Ac] \times 100$$

Where A_c is absorbance of control and A_s is absorbance of sample.

Free radical scavenging by using ABTS (2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) method

To determine ABTS* free radical scavenging activity of extract, method of Sellappan and Akoh's was followed with slight modification. Equal volume of 7mM ABTS aqueous solution and 2.45mM $K_2S_2O_8$ incubate for 16 hours at room temperature to generate ABTS* free radicals. Absorbance was determined at 734nm with 95% ethanol as control. Different concentrations of extract were prepared by dissolving 25, 50, 75, 100μL of extract with DMSO so that total volume of each solution will be 1000μL. Then 0.2mL of each sample was mixed with 2mL of ABTS solution and absorbance was noted at 734nm (Sellappan and Akoh, 2002).

Determination of Total Phenolic Content;p

To determine total phenolic content of methanol extract of *Nigella sativa*, Folin-Ciocalteu reagent was used. Gallic acid was used as standard solution. Different concentrations of Gallic acid i.e., 1, 5, 10, 15, 20 ppm solutions were prepared from stock solution of Gallic acid (50mL) and diluted with distilled water up to the volume of 10mL. In 1mL of extract 5mL of Folin- Ciocalteu reagent was mixed and placed in dark for 5 minutes. After 5 minutes 4mL of 7.5% solution of sodium carbonate (Na_2CO_3) was added. Solution was incubated for 2h and then its absorbance was noted at 760nm. Absorbance of different concentrations of Gallic acid solution was also noted at the same wavelength. To plot calibration curve, average absorbance values of different concentration of Gallic acid solution were used (Singleton, 1999).

Determination of Total Flavonoids

Aluminum chloride colorimetric method was used to determine total flavonoids. 5mL of methanol extract of black cumin seeds were taken in test tube. In test tube 0.3mL of 5% sodium nitrate solution was added. After 5 minutes 0.3mL of 10% $AlCl_3$ mixed and placed for 6 minutes. After 6 minutes 2mL of 1M sodium hydroxide was added. At the end solution was diluted with distilled water so that total volume of solution would be 10mL. Solution was incubated for 15 minutes and then absorbance was noted at 510nm.

Rutin was used as a standard solution for determination of total flavonoids. Different concentrations of rutin solution 1, 5, 10, 15, 20, 25 ppm were prepared from stock solution. Absorbance of these solutions was measured in order to draw calibration curve (Atanassova *et al.,* 2011).

Results and Discussion

Phytochemical Screening (Qualitative)

Nigella sativa seeds have two different forms of alkaloid isoquinolide and pyrazol alkaloids. The volatile oil contained saturated fatty acids, saponin and alpha hederine (Forouzanfar *et* al, 2014, Kooti *et a*l, 2016). Phytochemical analysis of methanol extract of *Nigella sativa* shows the presence of alkaloids, terpenoids, steroids, phenols, flavonoids, saponins, diterpenes, carboxylic acid, coumarine and carbohydrates. While

emodol and pholabatannins were not found in methanol extract of *Nigella sativa* as showen in Table 1. Due to presence of these phytochemicals the importance of *Nigella sativa* in medicinal fields has been increasing day by day.

Table 1: Phytochemical constituents of methanolic extract of *Nigella sativa* seeds.

Constituents	**Reagent / test**	**Result**
Alkaloids	Mayer's test	+
	Wagner's test	+
	Hager's test	+
Terpenoids	Salkowski test	+
Phenols	Ferric chloride	+
Steroids	Acetic anhydride & H_2SO_4	+
Flavonoids	Alkaline reagent test	+
	Lead acetate test	+
Saponins	Froth test	+
Diterpenes	Copper acetate	+
Pholabatannins	1% HCl	_
Carboxylic acid	Sodium bicarbonate	+
Coumarine	Sodium hydroxide	+
Emodol	25% NH_3 solution	_
Carbohydrates	Molish's test	+

Detected = (+), Not detected= (-)

Biological Analysis

Antibacterial Activity

Antimicrobial power of methanol extract of *Nigella sativa* seeds was determined by using divergant bacterial strains. Different concentrations of extract was used in order to study the anti-bacterial activity more effectively against different bacteria as shown in Table 2.

Table-2: Antibacterial Activity of *Nigella sativa* extract.

Plant extract	**Bacterial culture**	**Inhibition zone(mm)**	**% inhibition**
Control (Amphotericin B	*S. aureus*	32	100
25µL		14	43
50µL		15	47
75µL		16	49
Control (Amphotericin B	*E. coli*	35	100
25µL		17	51
50µL		18	53
75µL		19	55
Control (Amphotericin B	*B. subtilis*	36	100
25µL		14	40
50µL		15	42
75µL		15.5	44
Control (Amphotericin B	*B. pumilus*	36	100
25µL		14	38
50µL		15	42
75µL		16	44
Control (Amphotericin B	*P. aeruginosa*	31	100
25µL		16	51
50µL		17	53
75µL		17.5	55

Moderate antibacterial potential was showen by methanol extract of *Nigella sativa* seeds against all stains of bacteria. Maximum zone of inhibition was showen by methanol extract of *Nigella sativa* against *E. coli* and *P. aeruginosa* where percentage inhibition is more than 50 %. While methanol extract of *Nigella sativa* showed small antibacterial activity against *B. subtilis* and *B. pumilus* bacteria.

Antiradical Scavenging
By DPPH Assay

The antioxidant potential of methanolic extract of *Nigella sativa* seeds was studied by using DPPH method as described in literature. In order to examine free radical scavenging of *Nigella sativa* seeds different concentrations of extract were prepared. The result is shown in Table 3 and Figure 2.

Table-3: Antioxidant activity of *Nigella sativa* by DPPH method.

Concentration of *Nigella sativa* (µL)	**%age inhibition**
25	29.54
50	37.9
75	39.9
100	48.9

Antioxidant potential of *Nigella sativa* seeds extract is concentration dependent. As concentration increases, percentage inhibition increases. When concentration of extract is 25μL percentage inhibition is 29.54%. At 50μL, the percentage inhibition is 37.9%, at 75μL percentage inhibition is 39.9% and at 100μL percentage inhibition is 48.9%.

ABTS Assay

The antioxidant potential of methanolic extract of *Nigella sativa* seeds was also studied by using ABTS method. Different concentrations of extract were prepared in order to determine antioxidant potential of sample. Result is shown in Table 4.

Table-4: Antioxidant potential of *Nigella sativa* by ABTS method.

Concentration of *Nigella sativa*(µL)	**%age inhibition**
25	28.84
50	35.02
75	45.87
100	54.94

The results show that antioxidant potential of extract of *Nigella sativa* is concentration dependent. The maximum antioxidant potential was shown by 100μL sample.

Phytochemical Screening (Quantitative)
Total Phenolic Content (TPC)

Total phenolic content of extract of *Nigella sativa* was determined by using Folin-Ciocalteu reagent in the presence of gallic acid as standard. Total phenolic content of sample expressed as gallic acid equivalent (GA) per mg. It is shown in Fig. 1.

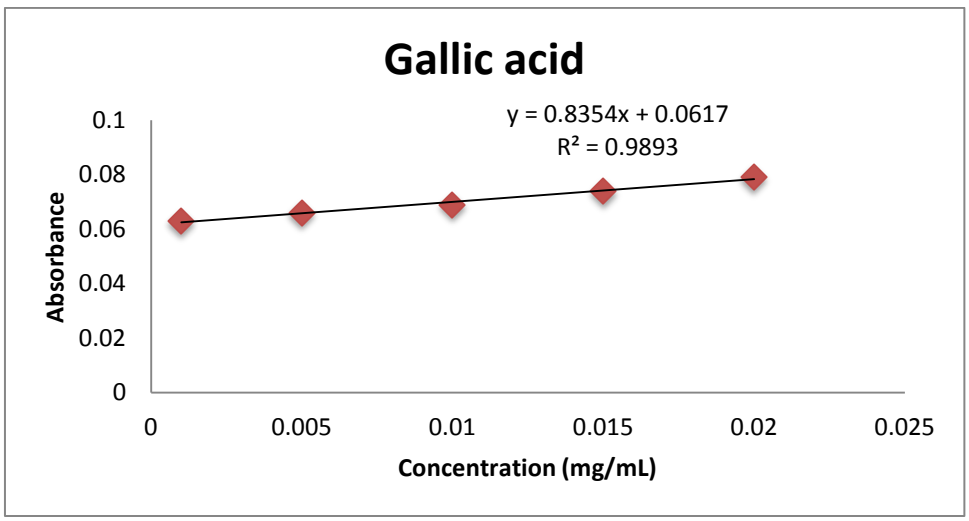

Fig.1. Calibration curve of Gallic acid.

Total phenolic content (TPC) of sample was calculated by using calibration curve and result was 4.39 ± 0.90.

Total Flavonoids Content (TFC)

Total flavonoids content of *Nigella sativa* extract was determined by using aluminum chloride ($AlCl_3$) reagent through spectrophotometric method. Total flavonoids content of extract was expressed in terms of rutin equivalent (mg of RU/ mL of extract). The result is shown in Fig.2.

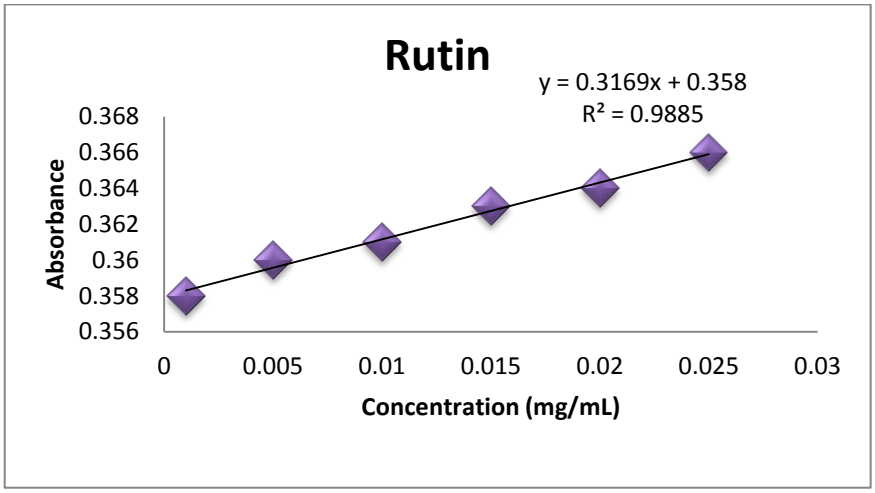

Fig.2. Calibration curve of Rutin.

Total phenol contents (TPC) and total flavonoids content (TFC) of *Nigella sativa* was calculated through calibration curve and was found that TPC and TFC of sample is 4.39 ± 0.90 and 10.65 ± 0.61 respectively.
Extracts of *Nigella sativa* seeds have ability to show anti-radical, anti-bacterial and phytochemical activities and very good results were obtained which were similar to Ajaib *et al.* (2016) while working on the *Andrachne cordifolia* for antimicrobial and antioxidant screening. Through phytochemical screening it was revealed that alkaloids, steroids, flavonoids, terpenoids, phenols, coumarine and carbohydrates are present in methanolic extract of *Nigella sativa* seeds while pholabotannins and emodol are absent these findings are similar to Ajaib *et al.* (2017) during phytochemical investigation of *Cocculus laurifoius*. Anti-bacterial activity was performed against *S. aureus, E. coli, B. subtilis, B. pumilus, P. aeruginosa* bacterial strains and maximum zone of inhibition was observed against *E. coli* and *P. aeruginosa* where percentage inhibition is more than 50 %. While methanol extract of *Nigella sativa* showed small antibacterial activity against *B. subtilis* and *B. pumilus* bacteria as reported by Maqbool *et al.* (2017) during investigation of antibacterial activity of *Cirsium arvensis*.

Methanolic extract of *Nigella sativa* seeds were also screened for anti-radical scavenging activity by using DPPH and ABTS methods. Total phenolic contents (TPC) and total flavonoid contents (TFC) were determined in quantitative screening and significant results was obtained which are almost similar to Ajaib *et al*. (2013) report on antioxidant screening of *Rivina humilis*

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