

Biological Activity of '3, 3', 4', 5, 7-Pentahydroxyflavone Isolated from Green Leafy Vegetables of Karnataka on three Bacterial Strains

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<http://dx.doi.org/10.13005/bpj/1951>

(Received: 17 February 2020; accepted: 09 April 2020)

Screening of antibacterial compounds helps to combat the resistance exhibited by bacteria to antibiotics. The aim of this study is to isolate '3, 3', 4', 5, 7-pentahydroxyflavone' (quercetin) a well-known flavonoid from edible greens of Karnataka with a focus on its biological activity. We employed column chromatography for successful extraction of compound, identified it by High Performance liquid chromatography (HPLC), UV-Visible Spectroscopy and Infra-red Spectroscopy. For knocking antibacterial action, we used five methods, the first method is screening of antibacterial activity by Agar well-diffusion, second is determining anti-bacterial motility, third is biofilm formation control, fourth is determination of release of absorbed materials and fifth is DNA fragmentation assay. The results of all the methods employed were complementary with minimal concentration of compound i.e. 10 µg/ml on all the tested bacteria. Induction of DNA fragmentation with 10 µg/ml of the compound is an important observation of the presented study on all the pathogens. Hence, identification of new unexploited sources of flavonoids from edible greens helps in combating emerging drug resistance. To conclude, the exploitation of ample sources of '3, 3', 4', 5, 7-pentahydroxyflavone' from commonly preferred food increases its consumption rate thereby preventing the risk of infections.

Keywords: Anti-Bacterial; Chromatography; Identification; Phytochemicals; Quercetin; Spectroscopy.

India is a richest source of biodiversity with 2500 plant species, out of which 100 species are recognized as traditional healers. World Health organization (WHO) estimates that 80% of the world's population rely on plants for their primary health care needs¹. Hence, knowledge of chemical composition of plants is necessary for understanding the possible mechanism of action, led to focus on

principle components of plants in contributing medicinal properties. Phenolics, the broad class of secondary metabolites are ubiquitous in plants with flavonols, flavanones and flavonol subgroups. These subgroups are continuing to receive ample attention due to their vast biological potential. Four kinds of flavonoids are broadly investigated for clinical use that include polyphenols derived from



tea, 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) and its molecular cousins. Due to massive attention on flavonoids by many investigators, today, 3, 3', 4', 5, 7-pentahydroxyflavone, a flavonol abundant in foods has become the most studied flavonoid². The common name of well-known bioflavonoid, 3, 3', 4', 5, 7-pentahydroxyflavone is quercetin. The presence of these polyphenolic bioflavonoids is responsible for therapeutic potency of plant that contributes antioxidant action by scavenging mechanisms³. This has drawn our attention in identifying natural antioxidants with antimicrobial potencies from locally available greens. *Raphanus sativus L.* (radish) and *Anethum graveolens L.* (dill weed) are widely grown and used vegetable of South East Karnataka. The underground tuber of radish is exhaustively used and the leaves are very well neglected. Hence, in the present study, leaves of radish and dill weed have chosen for flavonoid and compared its biological activity.

R. sativus L. is an annual vegetable belongs to Brassicaceae family with an annual average yield of 42,276 tonnes. It has traditional importance in countries like China, Japan, Korea and Southeast Asia due to its adaptive ability, high yield and more nutritional value⁴. Root is consumed as vegetable; in some places, leaves are used in salads and garnishes. The seed, root and leaf of *R. sativus* are claimed to have several medicinal properties⁵. Raphani semen (dried ripe seed) is used in Chinese traditional medicine to promote digestion, treat dyspeptic retention, constipation, diarrhoea, breathless condition, dysentery and cough with phlegm⁶⁻⁷. Even though the medicinal use of Raphani semen has historical importance more than a thousand years, there is no literature available on the flavonoid content of leaves and identification of effective flavonol, 3,3',4',5,7-pentahydroxyflavone for its pharmacological use.

A. graveolens L. is an annual herb belongs to family Apiaceae. It is grown widely in Eurasia and in Karnataka and spread widely in Bijapur with a yield of 646 tonnes per year⁸. In Unani medicine, it is used in curing digestive problems and also used in more than 56 Ayurveda preparations like Maharasnadi kashayam, Mrithasanjeevani, Gugguluthiktaquatham and Sraswatharishtam. It was tremendously used by Egyptian doctors from over 5000 years

ago⁹. The pharmacological importance of *A. graveolens* is known for its anti-microbial, anti-hyper lipidemic and anti-hyper cholesterolemic activities¹⁰. Furthermore, the fruit is used in treating gastrointestinal disorders¹¹. Since, the plant has medicinal importance and there is a gap in literature on the 3, 3', 4', 5, 7-pentahydroxyflavone content of leaves of the plant, exploring its phytochemical components contributing in medicine is essential. Hence, the objectives of the study were screening of phytochemicals, identification and structural elucidation of flavonol, 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) from leaves of *A. graveolens L.* and *R. sativus L.* and exploring its biological potency.

MATERIALS AND METHODS

Collection of Material

The seeds of *R. sativus* and *A. graveolens* were grown in pesticide free environment, leaves were collected after 2-3 day water stress shade dried, powdered and stored. The authentication of the plant samples with voucher specimen number of *R. sativus* (2232) and *A. graveolens* (1236) were provided by Dr. Madhava Shetty, Assistant Professor, Department of Botany, S.V. University, Tirupathi.

ATCC strains of *Pseudomonas aeruginosa* (27853), *Staphylococcus aureus* (25923) and *Escherichia coli* (25922) were obtained from HI media Laboratories, India. All others chemicals and reagents used were of analytical grade obtained from Sigma Aldrich.

Extraction and Phytochemical Analysis

Adopted protocol was same for both the plants. About 25 g of leaf powder was taken in 100 ml of ethanol and was subjected to constant shaking at 120-130 rpm for 48 hours at room temperature. After 48 h, the extracts were filtered using Whatmann No. 1 filter paper and the solvent was evaporated until dryness. About 12g of dried extract was obtained from both the plant materials and stored at 4°C for further analysis.

Phytochemical analysis was performed for both the leaf extracts according to standard protocol and total flavonoid content was determined by Aluminium chloride method¹².

Purification of 3, 3', 4', 5, 7-Pentahydroxyflavone

Column chromatography was performed

according to the procedure of Chandrappa et al¹³. The concentrated plant compound obtained from column chromatography at 1mg/ml was subjected to HPLC along with the standard quercetin (3,3',4',5,7-pentahydroxyflavone) to check purity of the compound.

Characterization of 3, 3', 4', 5, 7-Pentahydroxy flavone

Mass Spectrometry-Based Multiple Reaction Monitoring (MS-MRM)

The qualitative analysis of the metabolite, 3, 3', 4', 5, 7-pentahydroxyflavone, was carried out using Multiple Reaction Monitoring (MRM) assay using QTRAP 6500, AB-SCIEX mass spectrometer. The samples were diluted to 1: 5 in 0.1% formic acid in water and analyzed in MS1, MS2 and MRM modes for the presence of 3,3',4',5,7-pentahydroxyflavone using the selected ion pair transitions¹⁴.

H 1 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is primarily related to the magnetic properties of certain atomic nuclei; notably the nucleus of the hydrogen atom and will demonstrate the number of atoms in neighbouring groups. Hence, in the present study, it was used as one of the tools for 3, 3', 4', 5, 7-pentahydroxyflavone identification¹⁵.

UV-Vis Spectrophotometry and FTIR Measurements

Spectrophotometric analysis of the isolated 3, 3', 4', 5, 7-pentahydroxyflavone was performed by UV-Vis Lambda 20 Spectrophotometer between 200 and 700 nm wavelength with a band width of 2 nm. Infrared absorption (IR) spectrum of flavonol 3,3',4',5,7-pentahydroxyflavone was recorded with Fourier Transform Infrared (FTIR) absorption spectrometer. The spectrum was recorded in the region between 4000 cm⁻¹ and 400 cm⁻¹¹⁶.

Determination of Biological Activity

Antibacterial Activity

Antibacterial activity of 3, 3', 4', 5, 7-pentahydroxyflavone extracted from both the plant species was detected primarily by agar well diffusion method by using ATCC strains of *Pseudomonas aeruginosa* (27853), *Staphylococcus aureus* (25923) and *Escherichia coli* (25922). 3, 3', 4', 5, 7-pentahydroxyflavone was prepared in 10 µg/ml, 20, 30, 40, 50, 100, 150, 200 and 250 µg/ml concentrations and checked for antibacterial

activity. The same strains of bacteria were also tested with the high-end antibiotic discs by performing antibiotic susceptibility test on nutrient agar plates. The zone of inhibition was measured for both the experiments. All the experiments were repeated thrice and results were noted¹⁷.

Determination of Motility Assays

Swarming, swimming and twitching motility of bacteria were detected with minimal to maximum concentration of 3, 3', 4', 5, 7-pentahydroxyflavone extracted from two plants. The appearance of spreading zones from the point of inoculation from the bottom of petri dish is noted for all the assays and results were noted¹⁸.

Effect of 3, 3', 4', 5, 7-Pentahydroxyflavone on Biofilm formation

Anti-biofilm activity of 3, 3', 4', 5, 7-pentahydroxyflavone was performed with 10 and 100 µg/ml concentrations of plant extracts by the method described by Lee¹⁹. Average of the three biological replicates was calculated for the percentage of biofilm inhibition by using the following equation.

$$\text{OD of test} / \text{OD of control} \times 100$$

Measurement of Absorbing Materials and Proteins

Fresh bacterial culture was inoculated in nutrient broth and cells in log phase were collected by centrifugation at 5000 X g for 15 min. The collected cells were washed three times with 0.1 M PBS (pH 7.4) and re suspended in PBS. Cell suspension of 100 ml was incubated at 37°C in shaking incubator for 4h in treatment with 10 and 100 µg/ml concentrations of 3,3',4',5,7-pentahydroxyflavone extracted [control, 1x MIC and 2x MIC]. The suspensions were removed from shaking incubator after 4h and centrifuged at 6000 X g for 5 min. The supernatants were read at 260 nm for 4 h and 24 h. Along with; the concentration of proteins in supernatant was assessed by using Lowry method. Absorbance was measured for three replicates at 280 nm²⁰.

DNA Fragmentation Assay

DNA fragmentation assay was performed according to standard procedure²¹. About 10⁴ cells of all the bacteria were treated with 10 and 100 µg/ml concentrations of 3, 3', 4', 5, 7-pentahydroxyflavone extracted for 4h, centrifuged at 5000 rpm for 5 min

and discarded the supernatant To the pellet, 20 μ l of TES (1 M Tris, 20 mM EDTA and 1%SDS) was added to lyse the cells and 10 μ l of RNase was added followed by incubation for 1h at 37°C. Added 10 μ l of proteinase K, incubated at 50°C for 1 hr and 30 min. The resultant solution was loaded onto 1% agarose gel and documented by using UV-trans illuminator (Biorad).

RESULTS

Phytochemical analysis of both the leaf extracts revealed the presence and absence of various phytochemical components which is shown in table 1. The flavonoid content of the leaf extract was found to vary between the two extracts tested. The exhibited concentrations after solvent extraction were 128 mg and 190 mg from *A. graveolens* and *R. sativus* respectively. The retention time of isolated 3, 3', 4', 5, 7-pentahydroxyflavone and the standard noted was the same as (Fig. 1). The mass/charge (m/z) ratio of isolated samples was in agreement with the standard (Fig. 2). The peaks of proton NMR of test samples and the standard were matching as shown in (Fig. 3). The UV- visible spectrum of both the samples showed maximum absorption from 230 nm to 450 nm which is similar to standard quercetin (3, 3', 4', 5, 7-pentahydroxyflavone) (Fig. 4).

The FTIR spectrum of quercetin extracted from *A. graveolens* and *R. sativus* were shown in fig 5 with its characteristic bands. The results of the FTIR were as follows; with respect to *A. graveolens* OH group stretches were found at

3409 and 3124 cm^{-1} , OH bending of the phenol function was detected at 1382 cm^{-1} . The C=O aryl ketonic stretch absorption was evident at 1664 cm^{-1} . C=C aromatic ring stretches were found at 1610, 1562, 1522 cm^{-1} . The in plane bending of C-H in aromatic hydrocarbon was detected at 1319 cm^{-1} and out plane bending bands were found at 941, 864, 852, 767, 724, 695, 639, 603 which were attributed to C-O stretch in the aryl ether ring, the C-O stretching in phenol and C-CO-C stretch and bending in ketone respectively. Where as in *R. sativus* OH group stretches were found at 3414 cm^{-1} , OH bending of the phenol function was detected at 1383 cm^{-1} . The C=O aryl ketonic stretch absorption was evident at 1660 cm^{-1} . C=C aromatic ring stretches were found at 1611, 1563, 1523 cm^{-1} . The in plane bending of C-H in aromatic hydrocarbon was detected at 1320 cm^{-1} and out plane bending bands were found at 942, 865, 853, 768, 725 respectively which were attributed to C-O stretch in the aryl ether ring, the C-O stretching in phenol and C-CO-C stretch and bending in ketone group. These results confirmed that the compound extracted is 3, 3', 4', 5, 7-pentahydroxyflavone.

3, 3', 4', 5, 7-Pentahydroxyflavone as a Potent Bactericidal Agent

The antibacterial activity of 3, 3', 4', 5, 7-pentahydroxyflavone extracted from both the leaf materials were promising.

In case of 3, 3', 4', 5, 7-pentahydroxyflavone from *A. graveolens* zone of inhibition (ZOI) was from 10 $\mu\text{g/ml}$ to 250 $\mu\text{g/ml}$ concentration, maximum zone being 22 mm was noted at 250 $\mu\text{g/ml}$ concentration on *S. aureus*, which is streamlined with Cefoxitin, Vancomycin and Ciprofloxacin. For *P. aeruginosa*, the maximum inhibition zone was 20 mm for 250 $\mu\text{g/ml}$ concentration which is in coordination with antibiotics Piperacillin, Ceftazidime and Imipenem. Similarly, the ZOI was 18 mm for both the test compounds and the antibiotics (Ampicillin, Sulphomethazole and Chloramphenicol) used for *E. coli*.

In case of *R. sativus*, 24 mm ZOI was exhibited by 3, 3', 4', 5, 7-pentahydroxyflavone for *S. aureus* which is in consistent with antibiotics *Penicillin* and *P. aeruginosa* exhibited 20 mm which is consistent with Piperacillin and Imipenem. In case of *E. coli* 3, 3', 4', 5, 7-pentahydroxyflavone exhibited highest (27 mm) among the aZOI when

Table 1. Phytochemical content of *R. sativus* and *A. graveolens* leaf extracts

Name of the Phytochemicals	<i>R. sativus</i>	<i>A. graveolens</i>
Phenolics	Present	Present
Flavonoids	Present	Present
Proteins	Present	Absent
Glycosides	Present	Present
Terpenoids	Absent	Present
Alkaloids	Present	Present
Cardiac glycosides	Mildly present	Present
Tannins	Present	Absent
Gums and Resins	Absent	Absent
Saponins	Absent	Absent

compared to the antibiotics tested and put (Tables 2 and 3).

3, 3', 4', 5, 7-pentahydroxyflavone shared antibacterial activity more or less similar to the tested antibiotics and in case of *E. coli* 3, 3', 4', 5, 7-pentahydroxyflavone extracted from *R. sativus* exhibited potent inhibitory zone than all the high-end antibiotics tested. It is reported from the literature that the antibiotics used for broad spectrum antibacterial activity like Cefoxitin, Vancomycin, Piperacillin, Cefazidime, Ampicillin and Imipenem inhibited cell wall synthesis and disrupted cell membrane²².

The obtained results by 3, 3', 4', 5, 7-pentahydroxyflavone extracted from both the plants which are on par with the results

of antibiotics, might be exhibiting the similar mechanism of action which is depicted in figure 6. Hence further investigations are necessitated in order to rule out the exact mechanism of action of 3, 3', 4', 5, 7-pentahydroxyflavone.

The prime requisite is to understand the action of any drug on microbial cell wall and membranes, the study is carried over for further parameter's assessment.

Limiting Bacterial Motility by 3, 3', 4', 5, 7-Pentahydroxyflavone

3, 3', 4', 5, 7-pentahydroxyflavone extracted from both the plant species restricted twitching motility better than the other two motility patterns at 100 µg/ml concentration in all pathogenic bacteria tested. In *E. coli*, 3, 3', 4', 5,

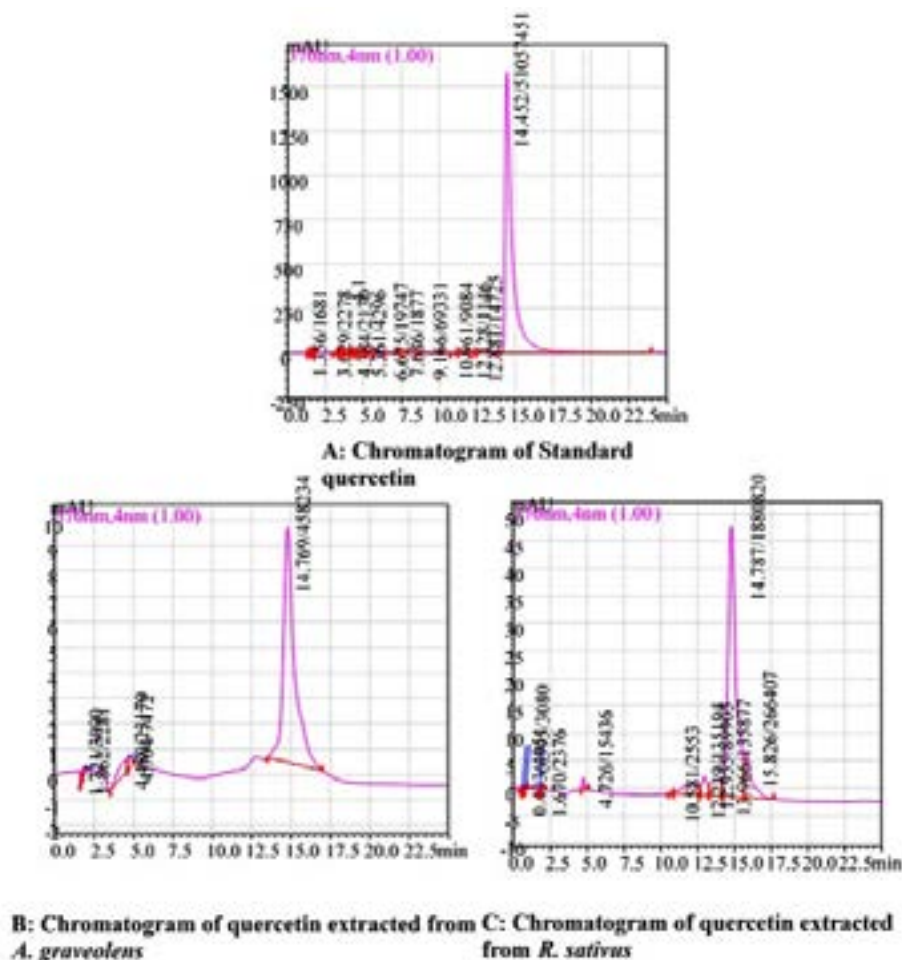


Fig. 1. HPLC chromatograms of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) extracted from plant sources in comparison with standard

7-pentahydroxyflavone extracted from *R. sativus* showed potent twitching motility restriction which is coping up with the results of antibacterial activity.

Whereas, 3,3',4',5,7-pentahydroxyflavone from *A. graveolens* presented good results than 3,3',4',5,7-pentahydroxyflavone from *R. sativus* on *P.aeruginosa* and *S.aureus* (Figure 7).

Regulation of Biofilm Formation by 3, 3', 4', 5, 7-Pentahydroxyflavone

The formation of biofilm is an important tool for developing resistance in bacteria. Hence, control of biofilm formation is an interesting strategy. 3, 3', 4', 5, 7-pentahydroxyflavone extracted from both the plant species in the current study had similar action with respect to biofilm

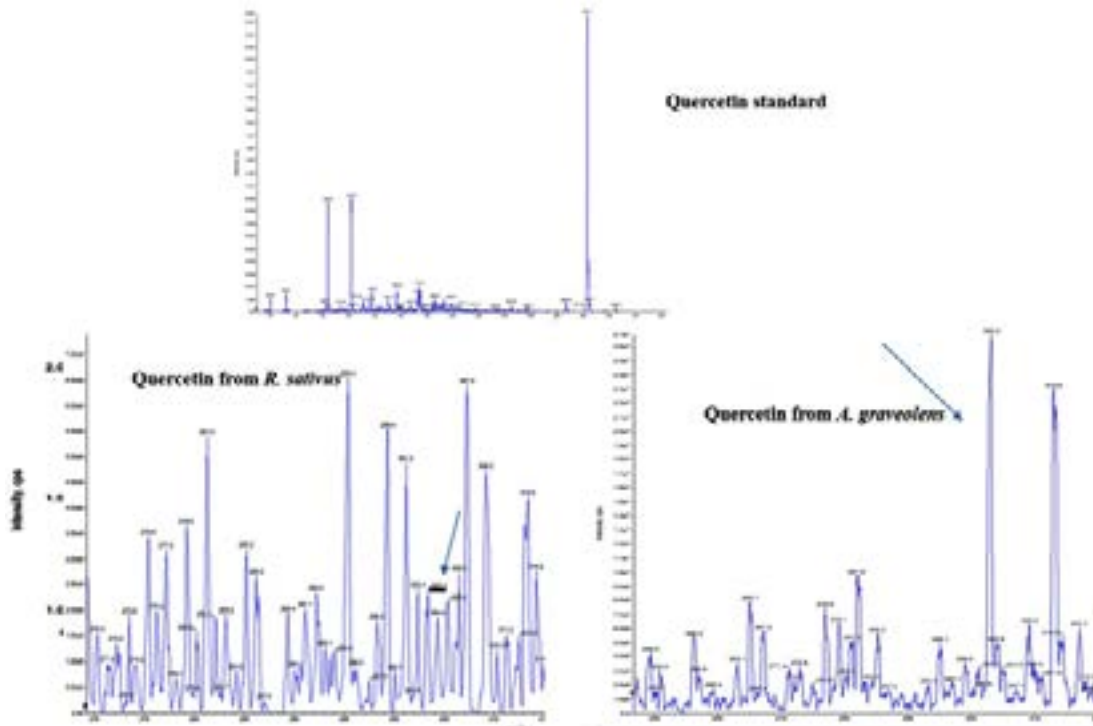


Fig. 2. LCMS analysis of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) extracted from plant sources in comparison with standard

Table 2. Antibacterial activity of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) extracted from *A. graveolens* and *R. sativus*

Concentration in µg/ml	<i>P. aeruginosa</i> (ZOI by quercetin)		<i>S. aureus</i> (ZOI by quercetin)		<i>E.coli</i> (ZOI by quercetin)	
	<i>A. graveolens</i>	<i>R. sativus</i>	<i>A. graveolens</i>	<i>R. sativus</i>	<i>A. graveolens</i>	<i>R. sativus</i>
10	06	06	06	06	06	06
20	06	06	06	6.5	07	07
30	07	6.5	07	6.5	08	7.2
40	07	07	08	7.5	09	7.5
50	7.5	07	10	8.5	12	10
100	08	08	14	12	14	13
150	9.5	09	16	15	16	18
200	13	12	18	18	17	23
250	20	20	22	24	18	27

formation exhibited by *S. aureus* than in case of *P. aeruginosa* and *E. coli*. Both the extracted compounds exhibited 90% biofilm inhibition on *S. aureus* (Figure 8).

3, 3', 4', 5, 7-Pentahydroxyflavone Impact on Release of Absorbing Materials and Proteins

The 260 nm absorbing materials are used as an indicative of damage to the membrane integrity. Any drug when explored as an antibiotic shall be its maximum efficacy with minimum concentration. Keeping this in view, we used minimum inhibitory concentration (MIC-10 µg/ml) and minimum concentration with maximum efficacy (100 µg/ml). The tested bacteria lost the cell constituents at 10 µg/ml and 100 µg/ml concentrations with 3, 3', 4', 5, 7-pentahydroxyflavone, indicating damage

to cytoplasmic membrane. As the permeability of cell membrane increases, leakage of intracellular proteins takes place in to surrounding environment thereby increasing the measurement of protein (Figure 9).

3, 3', 4', 5, 7-Pentahydroxyflavone Induced DNA Fragmentation

The shredding of bacterial genomic DNA is one of the early events of destruction. To test the cleavage of DNA, *P. aeruginosa*, *S. aureus* and *E. coli* were exposed to two different concentrations of (10 and 100 µg/ml) 3, 3', 4', 5, 7-pentahydroxyflavone extracted from both the plants and showed marked DNA damage after 24 hr incubation. The results confirmed that the extracted 3, 3', 4', 5, 7-pentahydroxyflavone

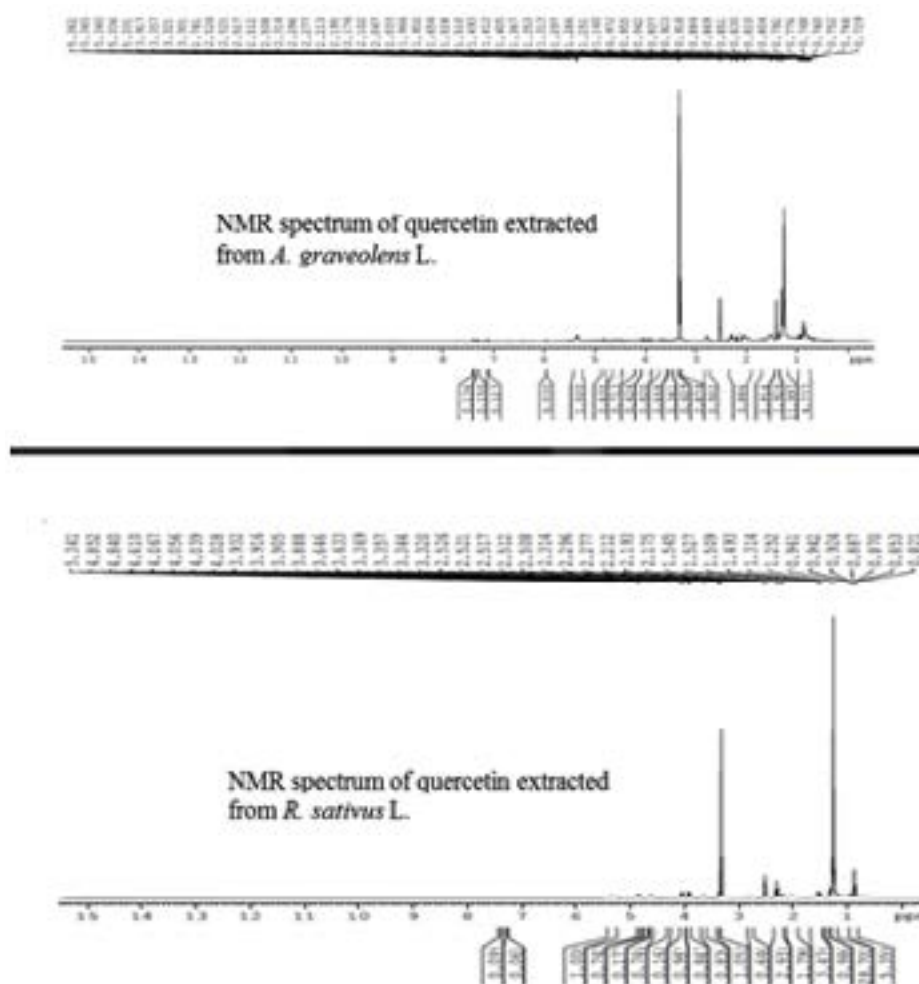


Fig. 3. NMR analysis of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) extracted from both plant sources

damaged the cell membrane and fragmented the DNA, thereby resulting in leakage of the contents and fragmentation of DNA (Figure 10).

DISCUSSION

Bacteria have three important components like cell wall, cell membrane and cytoplasm. Special

structures are also part of them like cilia, flagella and pili. Hence, in the present study mechanism of action of 3, 3', 4', 5, 7-pentahydroxyflavone on these structures were reported. The five methods employed for determination of antibacterial capacity of 3, 3', 4', 5, 7-pentahydroxyflavone components extracted from the two plants have

Table 3. Antibacterial activity exhibited by antibiotics on selected pathogens

<i>P. aeruginosa</i>		<i>S. aureus</i>		<i>E. coli</i>	
Antibiotic	ZOI (mm)	Antibiotic	ZOI (mm)	Antibiotic	ZOI (mm)
Piperacillin	20	Azithromycin	19	Ampicillin	18
Ceftizidime	19	Erythromycin	19	Cephalexin	23
Gentamycin	18	Ciprofloxacin	22	Amikacin	20
Tazobactam	21	Penicillin	25	Amoxycillin	22
Amikacin	18	Trimethoprim	16	Sepifime	19
Aztronam	24	Cefoxitin	22	Chloramphenicol	18
Ciprofloxacin	23	Tetracycline	18	Sulphomethoxazole	18
Imipenem	20	Vancomycin	22	Sepfuroxi	23
Levofloxacin	18	Gentamycin	16	Trimethoprim	21

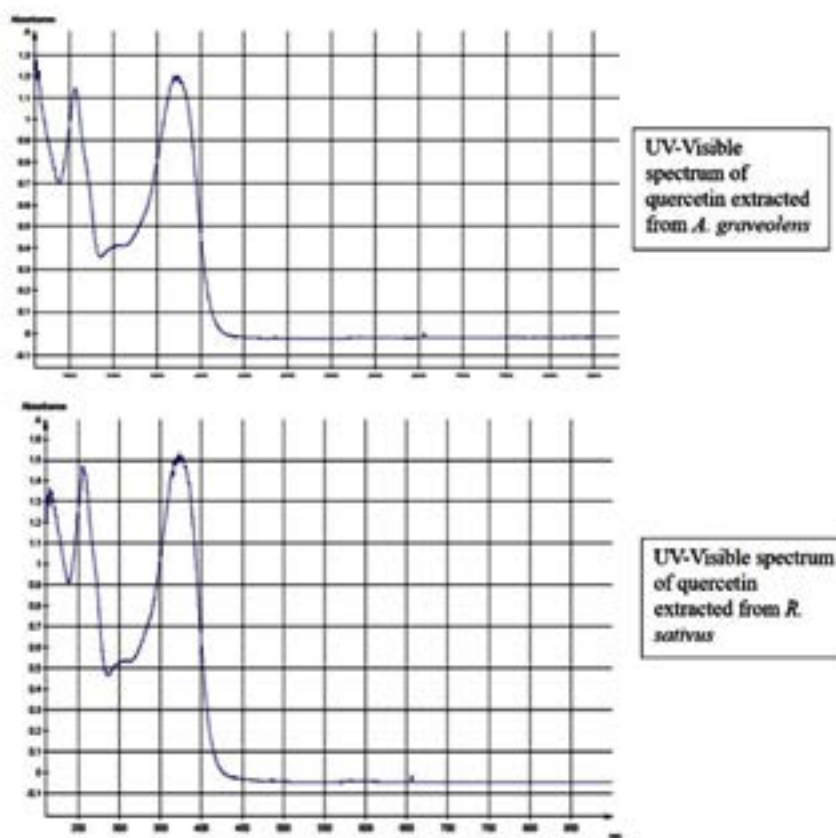


Fig. 4. UV-Vis analysis of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) extracted from plant sources

complementary results. Flavonols like quercetin, morin, rutin, myricetin and their derivatives show potent antibacterial properties. It was also confirmed in a study performed by Mary *et al.* where quercetin (3, 3', 4', 5, 7-pentahydroxyflavone) showed significant antibacterial activity on methicillin resistant *S. aureus*²⁶. Investigations made by Geoghegan, Wong and Rabie proved antibacterial activity at 0.0125 µg/ml are effective against *Porphyromonas gingivalis*²⁷. It was further stated that synergistic activity was shown when *S. epidermidis* (resistant to Amoxicillin) was exposed to combination therapy (quercetin and amoxicillin)²⁸⁻²⁹.

The above observations were in agreement with the results of the present study that 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) displays potent antibacterial activity on gram positive bacteria than on gram negative bacteria at minimal concentrations i. e 10 µg/ml. The reason behind its activity might be due to its cell membrane disruption nature as reported in the literature³⁰⁻³¹. Understanding the variations in the cell wall

structures and their modifications at the cellular level is an important approach in identifying antibacterial mechanism of any drug.

Bacterial motility plays a vital role in virulence by induction of various virulence determinants. As bacteria have outer appendages responsible for motility, it is therefore a goal to investigate the impact of 3, 3', 4', 5, 7-pentahydroxyflavone on motility patterns of bacteria i.e. swarming, swimming and twitching. Swarming and swimming are flagella mediated and twitching is type IV pilus mediated. Steps should be taken to prevent them, since these motilities elevate resistance to antibiotics. To our surprise, 3, 3', 4', 5, 7-pentahydroxyflavone tested in the present study restricted twitching motility more than the other two patterns of motilities³². And among the three organisms, twitching motility is potent in controlling the movement of *S. aureus*. Though *S. aureus* historically been regarded as non-motile bacterium, recent findings showed that it exhibits passive movement on agar surface by spreading. These spreading movements are due to branching

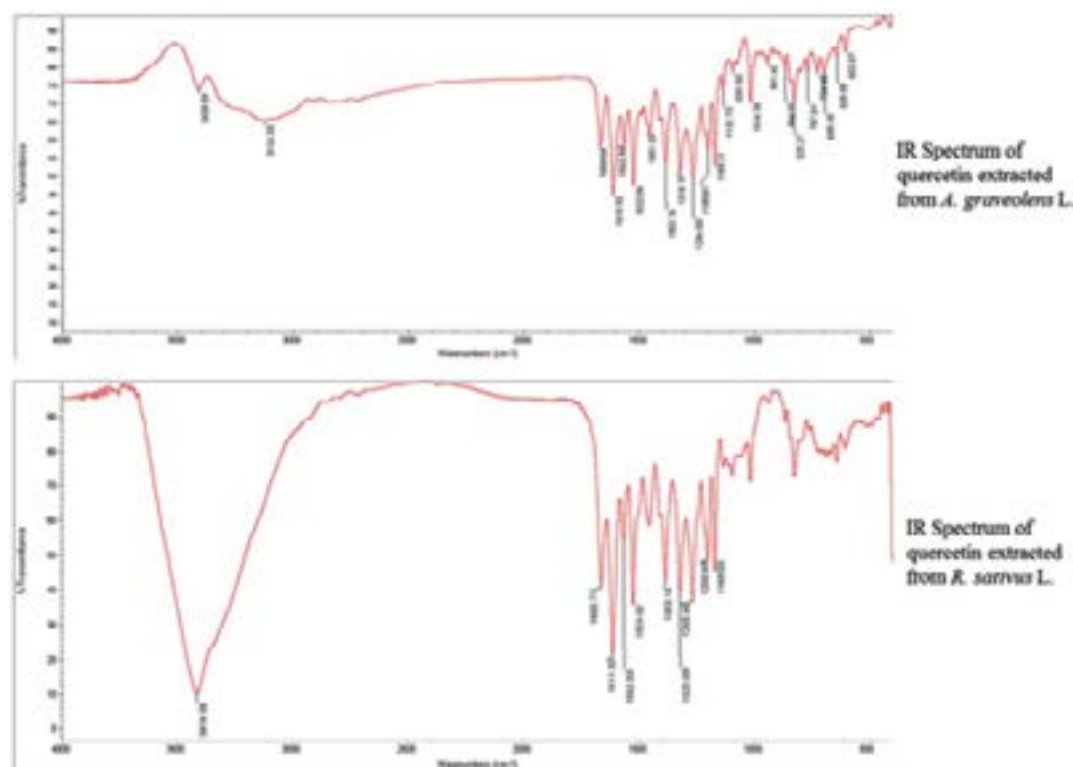


Fig. 5. FTIR spectrum of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) extracted from plant source

structures emerge from the central colony, called, dendrites. Research observations suggested that *S. aureus* is motile under certain circumstances³³. Twitching motility is considered as one of the important factors involved in the formation of biofilm on abiotic surfaces³⁴. Hence the control of twitching motility gains importance in the scientific research which is proven in the present study for the first time by 3, 3', 4', 5, 7-pentahydroxyflavone extracted from natural sources.

Furthermore, as the results of motility were encouraging, impact of 3, 3', 4', 5, 7-pentahydroxyflavone on bacterial biofilm were assessed. It is reported that biofilm producing organisms take a major share in causing bacterial infections; it acts as a protection barrier making the bacteria highly resistant to antibiotics³⁵. Controlling biofilm formation is an important strategy. To the best of our knowledge, the anti-biofilm potential of 3, 3', 4', 5, 7-pentahydroxyflavone extracted from *A. graveolens* and *R. sativus* is being reported for the first time in the study. The prior step in the formation

of biofilm relies on attachment of planktonic cells onto a substrate to form more sessile cells. The extent of inhibition of cell attachment by 3, 3', 4', 5, 7-pentahydroxyflavone extracted from the plants is quite encouraging in *S. aureus* than in *P. aeruginosa* and *E. coli*. Manner *et al.* demonstrated that flavonoids exhibited anti-biofilm activity against *S. aureus* which is in agreement with the present study³⁶. Apart from various strategies to control biofilm, novel strategies by using plants have to be reported to gain much significance³⁷⁻³⁸. The red wine flavonoid groups, quercetin, apigenin, chrysin and kaempferol showed biofilm inhibition of *S. aureus*³⁹. These are in consistent with present study results that 3, 3', 4', 5, 7-pentahydroxyflavone extracted from *A. graveolens* and *R. sativus* has similar biofilm inhibition on *S. aureus*.

Since 3, 3', 4', 5, 7-pentahydroxyflavone has motility and biofilm control effect, we thought of focusing on the measurement of released cell contents because of cell wall disruption. Measurement of released proteins and other

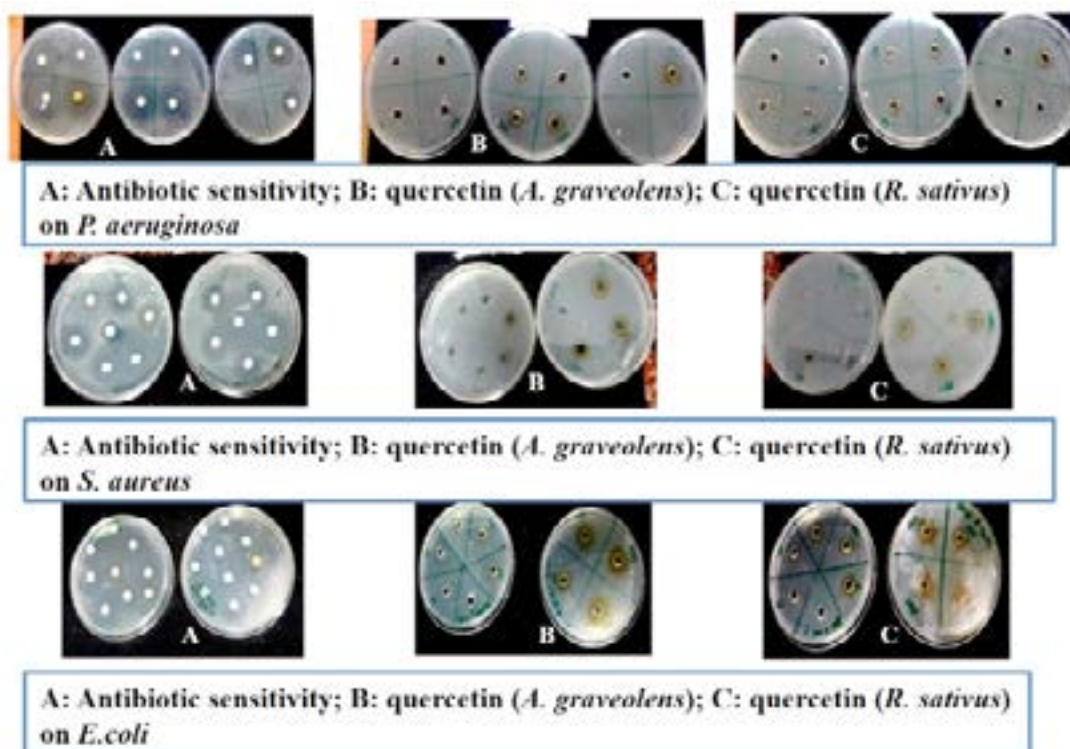


Fig. 6. Comparison of antibacterial efficacy of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) with antibiotics on three bacterial strains

materials from pathogenic bacteria treated with 3, 3', 4', 5, 7-pentahydroxyflavone were analysed. Bacterial cell absorbs key structural macromolecules that include proteins, nucleic acids and major amino acids (tryptophan, tyrosine and cysteine) at 260/280 nm⁴⁰. It is noted that as concentrations of 3, 3', 4', 5, 7-pentahydroxyflavone increased, soluble proteins from the superbugs, *S. aureus* and *E. coli* increased in the supernatant, resulting in leakage of cell membrane, thereby

decreasing the survival of the test organisms. Study performed by other investigators proved that leakage of cellular components at 260 nm and 280 nm were noticed when treated with spice extract essential oil against *S. aureus*. These observations also suggest that plant spices have cell membrane disruption activity which was in agreement with spice used in the present study *A. graveolens*⁴¹. Studies performed on black pepper essential oil also caused leaching of materials at

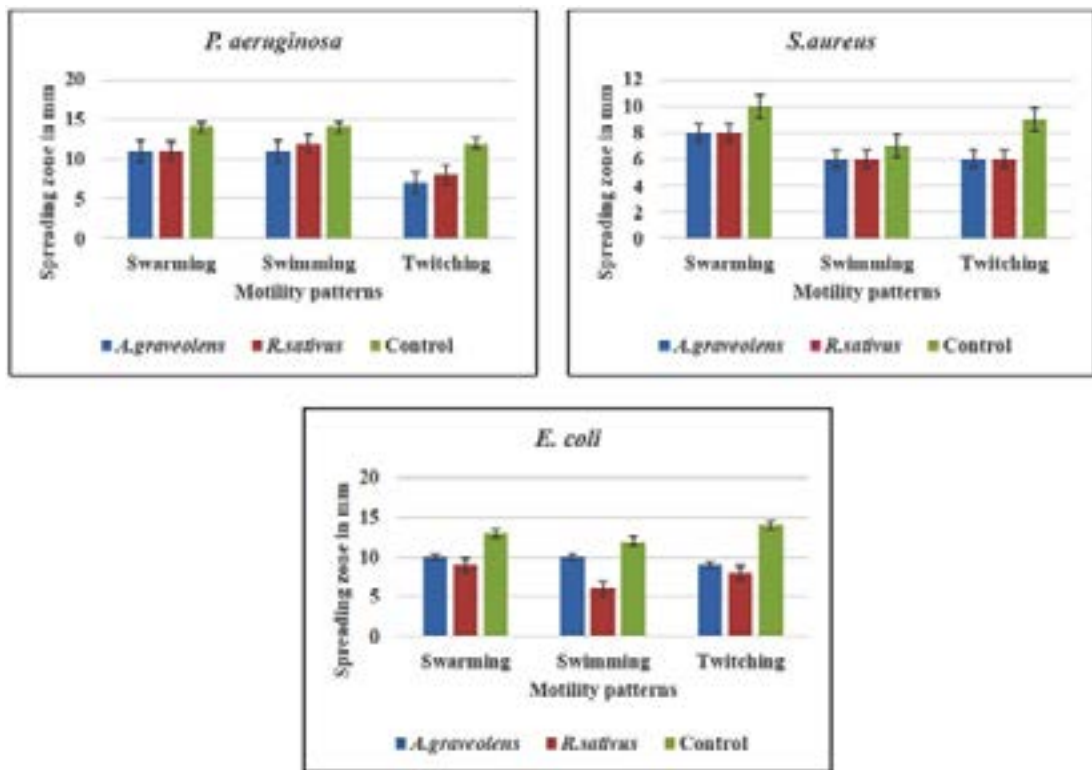


Fig. 7. Impact of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) extracted from plant sources on motility patterns

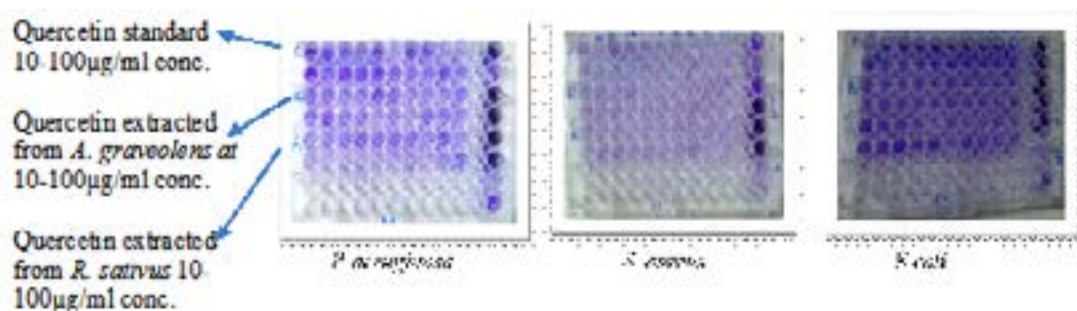


Fig. 8. Anti-biofilm activity of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) extracted from plant sources on Pathogenic microorganisms

260 nm and 280 nm against *S. aureus* indicating the membrane permeability⁴². These observations prove the fact that plant components possess potent phytochemicals that aid in disruption of cell membrane.

The effective role of 3, 3', 4', 5, 7-pentahydroxyflavone on released materials motivated to screen for its influence on genomic DNA. When tested on bacteria, 3, 3', 4', 5, 7-pentahydroxyflavone induced fragmentation of DNA. It was reported by Jose and his co-investigators that *E.coli* upon treatment with a chemical compound, hydrogen peroxide induced DNA fragmentation⁴³. Further Mori in his observations found that flavonoids are the strong

antibacterial agents that elicit DNA damage⁴⁴. Study by Ohemeg et al found that flavonoids were responsible for inhibitory activity of *E.coli* DNA damage and *Staphylococcus epidermis* which are in co-ordination with the present study results⁴⁵. These observations evidenced that flavonoids act on nucleic acid system and helps in proper functions of DNA, leading to growth inhibition. To our knowledge this is the first report to identify a compound responsible for DNA fragmentation in bacterial strains extracted from plant sources where as it is well studied in cancer cells.

To conclude 3, 3', 4', 5, 7-pentahydroxy flavone was extracted successfully by Column chromatography from the leaf extracts. The

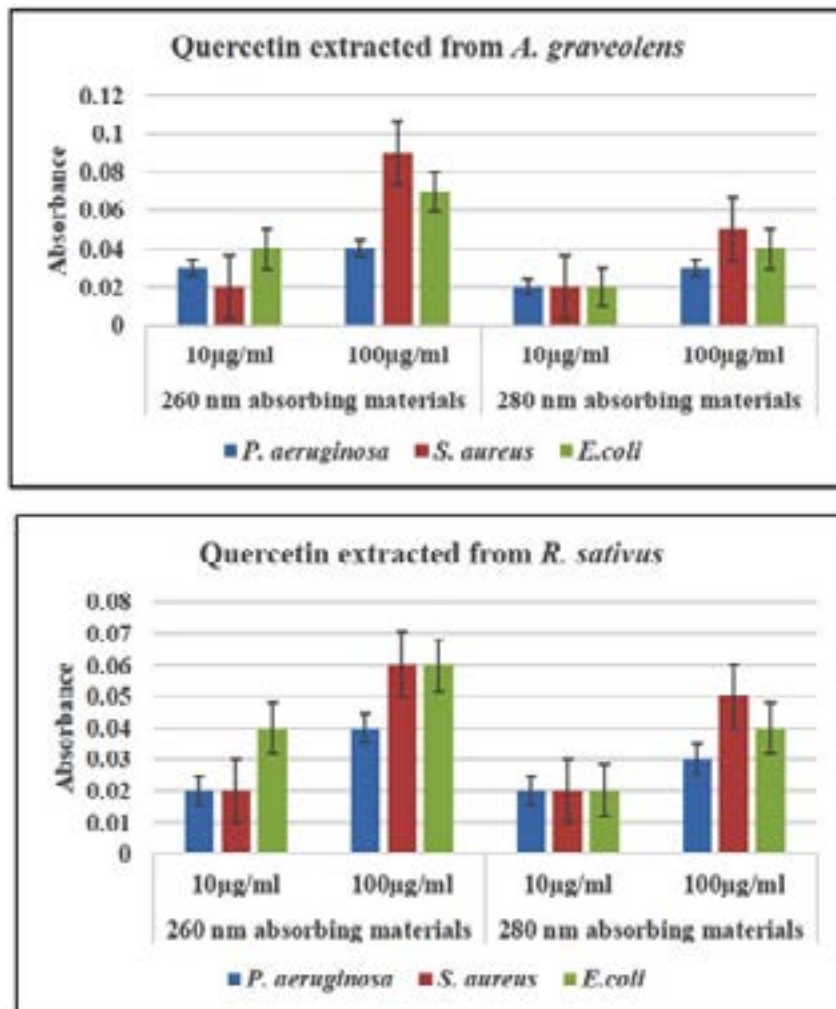


Fig. 9. Impact of 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) on release of proteins and nucleic acids from pathogenic microorganisms

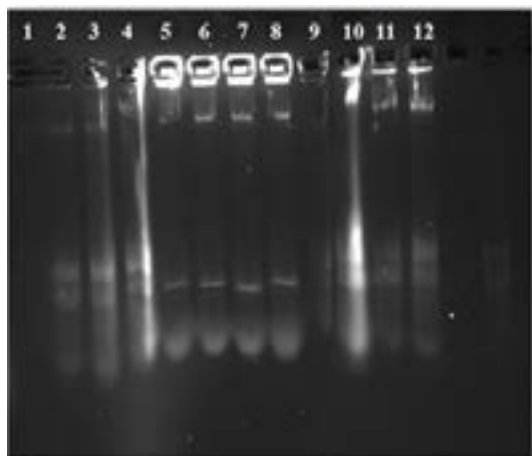


Fig. 10. 3, 3', 4', 5, 7-pentahydroxyflavone (quercetin) induces DNA fragmentation on tested bacteria. 1, 2: quercetin extracted from *A. graveolens* on *P. aeruginosa*; 3, 4: quercetin extracted from *R. sativus* on *P. aeruginosa*, 5, 6: quercetin extracted from *A. graveolens* on *S. aureus*, 7, 8: quercetin extracted from *R. sativus* on *S. aureus*, 9, 10: quercetin extracted from *A. graveolens* on *E. coli*; 11, 12: quercetin extracted from *R. sativus* on *E. coli*

proposed antibacterial mechanism of 3, 3', 4', 5, 7-pentahydroxyflavone extracted from plant source is as follows: attenuation of pathogenicity, restriction of twitching motility, reduction in biofilm formation, alteration in cytoplasmic membrane function, and induction of DNA fragmentation which are novel identifications from the selected plants.

ACKNOWLEDGEMENTS

The authors are grateful to Sri Devaraj Urs Academy of Higher Education and Research for proving all the infrastructure. The authors are thankful to Dr. Udaya Kumar and Miss Rajini from GKVK Bangalore for their support in HPLC analysis. The authors are also thankful to Dr. CV Raghuvier from Yenepoya Medical College, Mangalore for helping in Mass spectral analysis of the compound.

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