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Original Research Article

Extraction and isolation of flavonoids (quercetin) from tuberous part of Raphanus sativus (Radish)

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Abstract: Raphanus sativus L. also known as radish is a well-known edible vegetable that has been widely used as a dietary supplement and has a high reputation due to its rich phytochemical profile and related health promotion. The current study was done to extract, isolate, characterize, and determine quercetin in the tuberous part of Raphanus sativum in a systematic phytochemical and analytical system. Soxhlet extraction using the solvents of increasing polarity was carried out using dried tuberous roots after which preliminary phytochemical screening was done that established the presence of flavonoids and other secondary metabolites. The amounts of total flavonoid content were determined by the aluminium chloride colorimetric assay, with quercetin acting as a control on the assay; the findings showed that the ethanol extract had a greater amount of flavonoid in it, as compared to the ethyl acetate extract. The flavonoid-enriched fraction was then subjected to isolate quercetin using column chromatography and purity of the substance was observed by TLC and HPTLC methods. The chromatographic result was found to show that there is a close correlation between the isolated compound and the standard quercetin in terms of Rf and densitometric profile. The elucidation and confirmation of the structure was done using UV- visible spectroscopy, FTIR spectroscopy and a 1H -NMR analysis. The pure compound exhibited an optical absorption peak of about 370-375 nm, FTIR spectral characteristics that matched those an aromatic flavonol framework and proton NMR signals correlated with the aromatic and hydroxyl proton pattern of quercetin. Using quantitative analysis, it was found that quercetin contributes a large proportion of the total flavonoid content of the extract of radish tuber this paper substantiates the fact that the tuberous part of Raphanus sativus is a credible source of quercetin in nature and the effectiveness of the extraction and analysis approach employed. The results suggest that nutraceutical and phytopharmaceutical products including radish tubers may be included.

Keywords: Raphanus sativus (Radish), TLC and HPTLC methods, UV- visible spectroscopy, FTIR spectroscopy, FTIR spectral characteristics.

INTRODUCTION

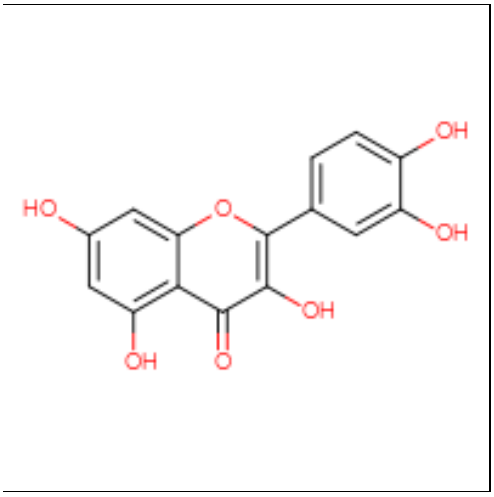
1.1. Phytochemical Importance of Raphanus sativus
Radish (Raphanus sativus L.) is a cuisine-varying plant that is highly consumed in Brassicaceae family. It is famous due to its complete nutritional balance, as well as due to a vast range of bioactivity secondary compounds. These include isothiocyanates -glucosinolates derivatives -along with a group of widely distributed polyphenols, phenolic acids, and flavonoids including quercetin and kaempferol, are the primitives' phytochemical components that characterize the biochemical profile of the plant1. Carbohydrates, essential element of minerals: especially potassium. These bioactive components provide potent antioxidant properties, which are reflected by the free-radical scavenging effects, lipid peroxidation inhibition as well as the enhancement of endogenous antioxidant defense mechanism2v. Therefore, the range of pharmacological effects that has been recorded in the scientific literature is based on such attributes.3

Radish extracts have been revealed to regulate a number of key pathophysiological mechanisms that are relevant of diseases like diabetes, neoplastic disease, hepatic damage, and inflammation4. This modulation is mainly done by the means of controlling redox status, up-regulation of detoxifying enzymes, and changes in glucose homeostasis and energy metabolism- both of which are supported by experimental studies as well as systematic reviews5.

One of the large subsets of the antioxidant, antidiabetic, anti-inflammatory, and cytoprotective activity of this phytochemical contains flavonoids, especially quercetin6. The multidimensional bioactivities of quercetin support the position of the compound as a key compound in the therapeutic capability of radish-derived extracts. Additionally, quercetin-enriched extracts derived using tissues of radishes such as roots, callus cultures, hairy root systems, are suggested to be a promising source of nutraceuticals and phytopharmaceuticals due to their improved bioactivity and proven health effects7.

1.2. Flavonoids as Functional Bioactive Compounds
The Flavonoid is a heterogeneous group of secondary polyphenolic metabolites, which are abundant throughout the plant kingdom. Over the years over ten thousand diverse structures have been arduously obtained of a wide range of botanical sources, such as fruits, vegetables, cereals, roots, medicinal herbs, tea, and wine, pointing up both their importance as component foods in the human diet and to the maintenance of health8.They are characterised by a canonical 15-carbon skeleton which forms a C6 -C 3 - C6 structure (with two aromatic rings, traditionally denoted A and B) attached by a heterocyclic pyran moiety (C). This architectural theme offers the scaffold as to which the various functional groups could be attached, thus giving the spectral diversities as such which were seen within the class9.

Depending on certain structural adaptation flavonoids are divided into seven major subclasses. The flavonol, exemplified by quercetin and kaempferol, maintain a 3-hydroxyflavone backbone having an unsaturated C2 C3 bond. The flavones, including apigenin, luteolin10, do not have the 3-hydroxyl group. Flavanones (such as naringenin) have their C2 C3 bond saturated. Lastly, the flavanols, or catechins, most prominently, epigallocatechin gallate, display a C3C4 bond that is saturated and different hydroxylation types, thereby providing different antioxidant capabilities.11



Quercetin

MATERIALS AND METHODS

2.1. Chemicals and Reagents

All the reagents and other ancillary solvents used in this study were obtained through authorized suppliers, and where necessary, of analytical grade to guarantee reproducibility. Among the notable solvents was ethanol, ethyl acetate and petroleum ether which were known to be of analytical purity. The petroleum ether was purchased in the foamed form with a boiling range of 40–60-degree C. Qualified standard quercetin that purifies to 95 per cent purity was added as the calibration compound in the chromatography. In order to determine the flavonoid, we prepared 10 per cent of ethanol spray reagent with AlCl3. Silica gel (60 120 mesh) was loaded into chromatographic column and on-sheet analysis was done on pre-coated silica gel 60 F 1/2 6 HPTLC plates (20 x 20 cm, 0.2 mm thick). The mobile phases used (toluene, ethyl acetate, and formic acid) were blended together in well measured percentages to allow optimal separation. Filtering Before the extraction, samples were filtered using Whatman Grade 1 qualitative filter paper and Soxhlet extraction was done using cellulose extraction thimbles.

In the next step, the plates were developed and scanned on a CAMAG HPTLC system with Linomat 5 applicator. The detection was done at 254nm and 366nm wavelengths which gave out the chromatogram data. All reagents were not purified, and glassware was all rinsed with a suitable solvent to avoid contaminating the reagent.

2.2 Collection, Authentication, and Processing of Plant Material

The present study obtained fresh tuberous roots of Raphanus sativus L. uniquely in the local cultivation sites and markets in the right time of the year. The reason was to ensure that healthy and mature tubers with no form of disease were picked hence eliminating any mechanically damaged or decayed material. The samples were immediately put in sterile ethylene bags

and recorded using a thorough collection metadata and then taken to the laboratory to be processed further.

Authentication of plant material

Taxonomic confirmation of the plant material gathered in this paper was done by Dr. K. Venkata Ratnam who is an Assistant Professor in the Department of Botany of Rayalaseema University, Kurnool, Andhra Pradesh, India. Dr. Ratnam named the taxon as a Brassicaceae (Cruciferae) species, which was representing a species of Raphanus sativus L. A voucher sample bearing the Voucher No. 888 which will be used in the future is deposited in consistency with the authentication certificate dated 20-09-2023. This standard assurance will guarantee the botanical accuracy and replicability of the current study.

Processing of plant material

The tuberous roots were authenthed after which their phytochemical integrity was purported under controlled laboratory conditions. The roots were initially sponged under a running tap water to take away soil and debris that was caught on the root and then sponged with a distilled water. Any moisture on the surface was blotted off with a piece of blotting paper.

Using an aluminium stainless knife, the cleaned tubers were sliced into small and similar bits. A hot-air oven with a temperature of 45 degree C was used to dry the sliced material to obtain a constant weight, so that the moisture is removed and the flavonoid reserves do not degrade at a high temperature. The dried substance was then ground in a laboratory grinder and sieved through a 60-mesh to a fine homogenous powder.

The powdered tuber sample was placed in an airtight and amber-coloured glass and stored in a cool and dry environment to be extricated and phytochemical analysed would follow. Each and every step such as collection date, drying conditions and weight of samples were adequately documented so that the work could be traced and reproduced.

2.3 Extraction of Flavonoids

The extraction used in extracting flavonoids in tuberous portion of Raphanus sativus L consisted of a strictly regulated and reproducible Soxhlet extraction procedure, which was designed to help ensure the recovery of moderately polar and polar flavonoid constituent but it had to retain its structure intact.

A pre weighed, dried, and then sieved (60-mesh) quantity of the finely powdered final tuber material was first weighed. The powder was then surrounded in Whatman filter paper, and placed in a cellulose extraction thimble, which was then placed into the primary chamber of the Soxhlet device. This made it easy to keep the solvent flowing continuously and proved to avoid any loss of the fine particulates during extraction.

The extraction was done in a step-wise manner, using solvents of successively increasing polarity. The first solvent chosen was ethyl acetate to extract semi-polar flavonoid and then ethanol was used to extract more polar constituents. To extract the product during each cycle, the solvent of choice was put (about) 250mL into a round-bottom flask which was attached to the Soxhlet apparatus and equipped with a condenser. The complete equipment was heated in a controlled heating mantle and left to reflux through the process in 8 or 10 hours, hence ensuring thorough extraction of medicinally active phytoconstituents that are soluble. The process of extraction was repeated until the solvent contained in the squeegee tube assumed the almost colourless appearance indicating the process was complete.

After the completion of every extraction, the corresponding solvent extracts were left to cool down and then concentrated under reduced pressure in a rotary evaporator. The traces of residual solvents were removed in the air by dry cleaning in a desiccator and produced semi-solid residues. The extracts were then dried and weight gain or percentage yield of extracts were allotted and the extracts were transferred to amber-labelled containers to ensure that the extracts were not exposed to the light.

The ethyl acetate and ethanolic extracts obtained were stored in low temperature awaiting additional phytochemical screening and chromatographic isolation and spectral characterization. During the extraction, great care was taken of the extraction conditions such as volume of the solvent, temperature and the period of the extraction in order to provide reproducibility and comparability of extraction across the experimental batches.

2.4 Preliminary Phytochemical Screening

Miniate phytochemical screening Preliminary screening: The preliminary screening was to be undertaken to determine the presence of primary and secondary metabolites in extracts of tuberous part of the plant, Raphanus sativus L. qualitatively. This is the first analysis to clarify the chemical characterization of the preparations and to justify the basis on further chromatographic separation of flavonoid constituents.

A series of proven qualitative scientists were applied to the extracts of ethyl acetate and ethanol, and the procedures were followed to the specifications reported in the pharmacognosy literature. All the assays were conducted in controlled laboratory parameters and all of the reactions were triplicated to achieve uniformity and reproducibility. Reagents and

solvents were subjected to fresh treatment and necessary solvent blanks were also included to reduce the chances of false-positive outcomes.

The presence of carbohydrates was observed by Molisch test which becomes violet in color after reacting. The use of Mayer and Wagner reagents was used to identify alkaloids with an appearance of cream or reddish-brown precipitates indicating a positive result. The screening of flavonoid was done using the alkaline reagent test as well as the lead acetate test, which are based on reactions between flavonoid hydroxyl groups and a given reagent to give a visible colour change or to precipitate. The Liebermann-Burhard reaction was used to identify steroids and Keller-Killani test was used to determine glycosides. The qualitative analysis obtained showed the availability of flavonoids, alkaloids, carbohydrates, steroids, and glycosides in both extracts. It is worth noting that the reactions were mostly more intensive with the ethanolic extract implying higher concentration of polar phytochemicals. These findings are in line with previous reports on the necessity to use ethanol as a solvent in extracting flavonoids-rich fractions in previous reports with respect to extracting flavonoids in the pelagic fungal species of Raphanus salivate results of the preliminary phytochemical screening are summarized in Table 1.

Table 1. Preliminary phytochemical screening of Raphanus sativus tuber extracts

Phytoconstituent	Test Performed	Ethyl Acetate Extract	Ethanolic Extract
Carbohydrates	Molisch's test	+	+
Flavonoids	Alkaline reagent / Lead acetate test	+	+
Alkaloids	Mayer's / Wagner's test	+	+
Steroids	Liebermann–Burchard test	+	+
Glycosides	Keller–Killiani test	+	+

Note: (+) Presence of phytoconstituent; (–) Absence of phytoconstituent.

2.5. Estimation of Total Flavonoid Content

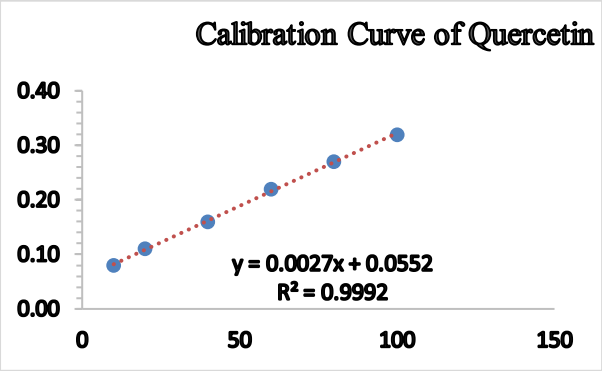
The amount of total flavonoid compounds in the tuberous part extracts of the plant species, Raphanus sativus, L. was determined using the aluminium chloride colourimetric assay, which is generally recognised to be a valid method of the quantitative estimation of flavonoids in botanical samples. This method relies on the fact that a stable yellow complex can be formed between the aluminium chloride and the keto- hydroxyl functional groups of flavonoids, followed by measuring it spectrophotometrically.

Concisely, the correct amount of each extract, which had been acquired in ethyl acetate and ethanol, was dissolved in the respective solvent to produce a working concentration. Sodium nitrite (5%(w/v) was also added to this solution and allowed to stand in five minutes. Aluminium chloride (10 per cent w/v) was then added and the mixture was left to incubate further after six minutes. Lastly, 1M sodium hydroxide was added to the reaction mixture and distilled water was used to bring the final volume to 100 mL. The mixture was mixed properly, and the absorbance was subjected to 510nm reading with the help of the UV-visible spectrophotometer, a reagent blank having been prepared in the same manner.

Quercetin was used as the reference standard used to build the calibration curve. Quercetin was used to develop the standard solutions at different concentrations, and the absorbance values were observed under the same experimental condition. A calibration curve was found to be liner in the concentration of quercetin verses absorbance within the sampled range. This was then used to obtain the total flavonoid content of the extracts as given in the regression equation that was obtained using the standard curve and was in milligrams of quercetin as the equivalent to one gram of dried extract. (mg QE/g).

The ethanolic extract exhibited comparatively higher total flavonoid content than the ethyl acetate extract, indicating that ethanol is a more efficient solvent for extracting flavonoid compounds from the tuberous part of Raphanus sativus. This observation correlates well with the preliminary phytochemical screening results and supports the

selection of the ethanolic extract for further isolation and characterization of quercetin.



2.6 Isolation of Quercetin

The separation of quercetin among flavonoid-rich extract of Raphanus sativus L. was carried out through the column chromatography followed by following it up with the use of thin-layer chromatography (TLC) method. Instead of exhaustive purification a simplified and reproducible methodology was chosen with the priority given to the acquisition of flavonoid fraction sufficiently pure to be subject together to spectroscopic confirmation12.

The stationary phase used in the column chromatography step was a clean and dry glass column filled with silica gel (60-120 mesh), which acts as a stationary phase13. The gel was first activated and then packed through the wet slurry method with the use of petroleum ether in ensuring the gel is packed uniformly and to avoid the occurrence of air bubbles. A representative sample of the dried extract (approximately 1g) was faintly adsorbed into a small amount of silica, dried to a free achieving powder and carefully heaped onto the column14. This was done gradually so as to maintain the integrity of the stationary state.

The gradient solvent system of steadily increasing polarity was taken through elution, whereby non-polar petroleum ether was used and where the mobile phase was progressively enriched with ethyl acetate, and then with ethanol. The gradual increase in polarity allowed the successful segregation of the constituents based on their different affinities to the stationary and the mobile phases. A periodic sampling of the fractions by placing them in clean and labelled test tubes was followed as well as checking on the basis of colour and clarity.

All of the obtained fractions were observed using TLC on silica gel plates previously pre-coated. The solvent system was toluene: ethyl acetate: formic acid (5: 4: 1 v/v/v) which gave a satisfactory flavonoid separation. The plates were air-dried and observed in UV light (254 and 366nm) after development. Further authentication was done with the help of spraying reagent of aluminium chloride and produced typical spots of yellow-fluorescence which testified the existence of flavonoid substances.

Fractions with similar TLC profile and Rf values close to the standard quercetin were combined. The combined fractions were diluted under a low pressure resulting in a yellowish solid residue15. The isolated compound had a Rf that matched the one of standard quercetin, and therefore, a successful isolation occurred. An isolated fraction was then taken through UV-Visible, and further spectroscopy on structural confirmation.

2.7 Identification and Characterization of the Isolated Compound

The pure flavonoid fraction of the isolated compound that was separated in the flavonoid-rich fraction of Raphanus sativus L. tuberous roots was systematically identified and characterized by the use of chromatographic and spectroscopic methods16. This step was an attempt to determine the chemical identity of the isolated molecule by comparison with reference standards and published literature data as opposed to using a single analytics method.

2.7.1 Thin Layer Chromatography (TLC) and HPTLC Analysis

The initial validation of the purified compound was carried out using TLC using silica gel 60 F_{254} plates silica as the stationary phase and ACCl2 as the mobile phase. The plates that were developed were viewed using UV light of wavelength 254nm and 366nm and the samples were then derivatised with an aluminium chloride solution. The separated pure compound appeared as a characteristic yellish spot fluorescent under UV 366 -nm exposure after sprayed with $AlCl_3$ which is typical of flavonol.

Additional confirmation was done by high-performance thin-layer chromatography (HPTLC) densitometry. The fraction isolated showed a sharp, symmetric peak with an Rf value of just under 0.38 which is in perfect agreement

with the Rf value of standard quercetin when analysed at the same chromatographic conditions17. The similarity of the values of Rf and profile of the peak with those of that of the reference standard was very close, thus showing strongly that quercetin is the main flavonoid constituent of the sample.

2.7.2 UV–Visible Spectroscopic Analysis

The electronic absorption properties of the obtained compound were determined using UV Visible spectroscopy. A UV spectrum was characterized by a sharp absorption peak (λmax) at 370 375nm wavelength, which falls within the range of flavonol-type components and which is related to Band I reaction at the flavonoid system of the skeleton. The conjugated aromatic structure is linked to this absorption band that occurs via π→ π + transitions18.

The measured λmax was similar to reported UV spectral data of quercetin further confirming the identity of the isolated compound as flavonols. The lack of any further strong absorptiometry bands showed a decent level of purity.

2.7.3 Fourier Transform Infrared (FTIR) Spectroscopy

The functional moieties of the isolated compound were determined using Fourier -transform infrared spectroscopy. The FTIR spectrum showed significant absorption band in the range of 3390 -1 to 3390 -2, which is a result of the presence of hydrogen bonds between phenolic OH vibrations. Strong band at 1655 cm -1 was obtained because of the presence of C=O stretching in the Carbonyl group of the flavonoids19v.

There were definite C=C stretching vibrations at 1605 -1 -1510 -1 -1260 -1030 -1 were observed and the spectral range between 1260-1030 -1 showed C-O and C-O-C-strengths. These functional group absorptions correlate with already known profile of quercetin of FTIR and supports the existence of multiple hydroxyl groups and aromatic flavonol backbone.

2.7.4 ^{1}H-NMR Spectroscopic Analysis

Structural confirmation was further strengthened by ^{1}H-NMR spectroscopy recorded in DMSO-d_6. The spectrum exhibited characteristic downfield singlets at δ 12.48, 10.75, and 9.62 ppm, corresponding to phenolic hydroxyl protons, which are a defining feature of quercetin due to strong intramolecular hydrogen bonding20.

The aromatic proton region showed well-resolved signals at δ 7.68 (d, J ≈ 2 Hz) and δ 7.55 (dd, J ≈ 8, 2 Hz) assigned to H-2′ and H-6′ of the B-ring, respectively. Additional signals at δ 6.88 (d, J ≈ 8 Hz) corresponded to H-5′, while the A-ring protons appeared at δ 6.42 (d, J ≈ 2 Hz) and δ 6.19 (d, J ≈ 2 Hz), assigned to H-8 and H-6, respectively.

The overall proton distribution, multiplicity pattern, and chemical shift values closely matched reported ^{1}H-NMR data of quercetin, thereby confirming the flavonol skeleton and substitution pattern.

2.7.5 Overall Structural Confirmation

The combination of the chromatographic (TLC and HPTLC), UV-Visible absorption properties, FTIR functional group study and the representation of the detailed features of the characteristics of the ^{1}H-NMR spectrum collectively confirms that the identified isolate of the tuberous component of Raphanus sativus L. is quercetin. The moving average of the data given by the various modalities of the analysis provides strong and valid data in support of the effective separation and single out the quercetin as the major flavonoid constituent in the radish tuber.

2.8 Quantification of Quercetin

In the current study, the concentration of quercetin of the flavonoid-rich extract of root tuberous in the Raphanus sativus L. was measured using an analytical tool based on a calibration model, and this ensures accuracy and reproducibility. The estimation was done by comparing an analytical response of the isolated compound and a reference quercetin standard, in the same experimental conditions.

The stock solution of quercetin was also placed in ethanol to create a standard stock solution after which a series of working solutions with a sensitivity range were created by several serial dilutions. The colorimetric method used to analyse these standards was the aluminium chloride colorimetric method and the absorbance measured at the wavelength of 510nm. A calibration curve was developed whereby melted absorbance used against quercetin concentration showed a very good linearity at the selected range hence confirming the validity of the method in relation to a quantitative analysis21.

To analyze the sample, precision in weighting the known amount of the flavonoid rich extract and mixing it with ethanol to make a clear solution was taken. The colour development process that was used on the standards was also used on the sample solution. The resultant absorbance was taken under the same conditions and the corresponding concentration of quercetin was obtained using the calibration curve based on the regression equation.

HPTLC densitometry as a confirmatory quantitative method was used in addition to the spectrophotometric estimation. The highest Rf of the isolated compound at an Rf of known concentrations of the reference standard were determined and compared to the peak area of the isolated compound. The dual approach reduced the analytical bias and established the accuracy of the quantification outcomes.

The amount of quercetin was in milligrams of quercetin equivalent/gram of dried extract (mg QE/g). The findings showed that the tuberous section of the Raphanus sativus plant has a quantifiable and value-added quantity of quercetin, further confirming its contribution to the importance as an important dietary and pharmacologically valuable flavonoid resource.

2.9 Statistical Analysis

All of the experimental determinations were carried out thrice, and the received data were represented in the form of mean with standard deviation (SD). To minimize inaccuracy, reproducibility, and variability during the extraction, identification and quantification processes, replicate measurements were conducted.

Linear regression analysis was used to produce calibration curves of quercetin which were assessed by the correlation coefficient (R2). The calibration plots with good linearity in the chosen concentration range were only used in quantitative estimation. These standard curves were used to come up with regression equations of sample concentrations.

The data processing, graph plotting, curve fitting, along with the construction of the calibration curves, UV to visible spectra, FTIR plots, HPTLC densitograms and regression analysis was done using OriginPro software. Peak identification and visual comparison of the sample and standard profiles was also done using the software.

To statistically analyze and validate numerical data, the SPSS software v22 was deployed. The descriptive statistical tools that could be used in SPSS were used in finding the values of means, standard deviations, and also to establish the consistency of data among replicates. This was a combination of graphical and statistical software that made the interpretation and presentation of the experimental results reliable.

The statistical tools applied gave a strong analytical tool in measuring the extraction efficiency, the flavonoid contents, and quercetin levels in the tuberous section of Raphanus sativus L, thus justifying the conclusions made in the current study.

RESULTS AND DISCUSSION

3.1 Phytochemical Profile of Raphanus sativus Tuber Extract

Initial phytochemical evaluation of tuberious parts of Raphanus sativus L. showed a wide range of secondary metabolites, which confirms the richness of radish root and supports its use to conduct an intensive study on flavonoids.

The selection of pertinent phytochemical classes was observed as a quality of ethyl acetate and ethanolic extracts through qualitative screening, which reported flavonoids, alkaloids, carbohydrates, steroids and glycosides.

The presence of flavonoids was reproducibly observed in both extracts, but the positive reaction was much stronger in the ethanolic fraction, indicating that the flavonoid compounds resolvable by polar solvents, ethanol, are more effective in solubilizing flavonoid recruits of radish tubers, probably due to the presence of multiple hydroxyl motifs that define flavonols, quercetin. This increased flavonoid response in the ethanolic extract agrees with the previous findings of solvent-selective efficiency of extracting phenolic compounds of radish and other Brassicaceae species. Identification of alkaloids and glycosides implies that other bioactive compounds exist in the tuberous component of Raphanus sativus and these could be acting in concert to give this plant its pharmacological effects. These findings of the presence of steroids are additional instances that demonstrate earlier findings that radish roots are a source of structurally varied secondary metabolites. Carbohydrates and solvent polarity of extracts and solvent matrix interactions also affect solvent extraction by carbohydrates, which are more nutritionally oriented rather than chemically oriented.

The phytochemical profile that was determined in the present study is consistent with the available literature that refers to the radish roots as an excellent source of polyphenols and flavonoids, with quercetin commonly being one of the major flavonol. The inter-extract differences in phytochemical intensity can be explained by solubility of the solvents, the maturity of the plant bloom, geographical origin and post-harvest processing environments.

3.2 Total Flavonoid Content and Quercetin Yield

Quantitative determination of total flavonoid content (TFC) of the tuberous part extracts of the powder of Raphanus sativusL. was assessed quantitatively using aluminium chloride colourimetric assay using the quercetin as the reference sample. This theoretical and analytical process gives a reliable estimate of flavonoid levels based on complexation between the aluminium ions and the hydroxyl groups of flavonoids, hence creating a chromophore to be detected by the spectrophotometer.

The calibration curve, which was obtained on the basis of the utilization of the multiple standard concentrations of quercetin, demonstrated a high level of linearity throughout the selected-concentration-zone, hence confirming the suitability of the method in quantitative analysis. The regression equation and correlation coefficient resulted from the derived regression equation indicated a strong relationship between absorbance and the concentration that guarantees the accuracy and precision of estimation of flavonoid in the extracts investigated in the study.

Comparison of the extracts has indicated that the ethanolic extract which had a higher total flavonoid content compared to the ethyl acetate fraction. This finding has been confirmed by initial screening of phytochemicals and highlights the ability of ethanol which is a polar solvent to be much efficient to extract phenolic constituent of the radish tuber matrix. The high proportion of hydroxylated flavonols as in the case of quercetin further explains the high extraction, which is being witnessed in polar solvents thus explaining discrepancies between the two extracts.

The chromatographic analysis supported quantification of quercetin. High-performance thin-layered chromatography (HPTLC) densitometry revealed a distinct peak on an R f value equivalent to quercetin of the bacterial extract, thus confirming the presence of quercetin as the main flavonoid compound of the extract. The value of the peak area of the isolated compound compared to response curves of known quercetin concentrations revealed a significant effect of quercetin to the overall flavonoid content in the ethanolic extract.

Overall, a combination of the spectrophotometric estimate and chromatographic determination proves that the tuberous part of Raphanus sativus is a valuable source of flavonoids, and quercetin makes a considerable portion of the total flavonoid pool. Such results support those previously reported about the radish roots and also give sufficient support to the extraction and isolation plan used in the current study.

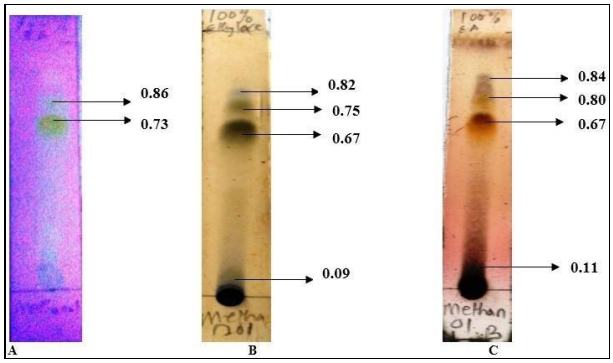
3.3 Chromatographic and Spectroscopic Confirmation

The identity and purity of flavonoid extracted through the tuberous part of Raphanus sativus L. were unambiguously proved through the joint use of both chromatographic and spectroscopic analyses. The use of various complementary methods helped to cross-verify the findings and significantly reduced the chances of false recognition which could be due to the usage of only one modality of analysis.

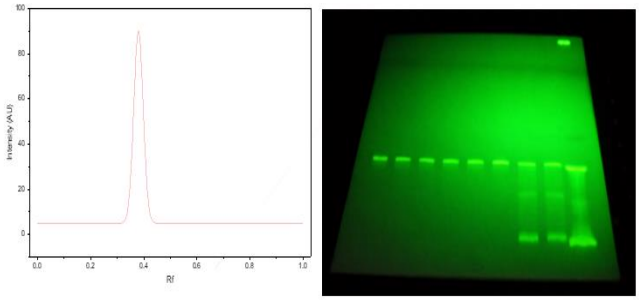
3.3.1 Chromatographic confirmation (TLC and HPTLC)

Initial identification of the isolate was done through the traditional thin-layer chromatography (TLC). Elution was done in a ternary solvent system and included toluene, ethyl acetate and formic acid in a proportion of 5:4:1 (v/v/v). Rf value of the isolated product was the same as that of a standard of quercetin and the spot was defined. With an 366nm illumination following the derivatization with aluminium chloride, there was a high intensity of fluorescence, a characteristic of the flavonol derivatives. Large spots would have indicated a lower purity in the sample, as the absence of added spots strongly suggested the high degree of purity was present.

This was followed up by the corroboration through high-performance thin-layer chromatography HPTLC. Densitometry scanning of the obtained plates revealed a sharp and symmetrical band of the isolate at the same R of the quercetin reference under the same analysis conditions. A near similarity in the R f values along with the identical profile of the peaks affirmed quercetin as the major flavonoid component in the extract. The slight differences in the baseline were charismatic thus showing that chromatographic isolation was quite efficient.



Representative column chromatographic separation of Raphanus sativus extract and TLC profiling of collected fractions showing flavonoid bands corresponding to quercetin under UV light and after $AlCl_3$ derivatization.



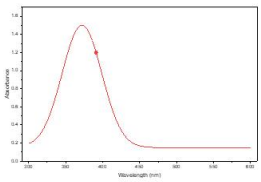
High-performance Thin-layer chromatography densitometric analysis of the extract showed that a well-resolved chromatographic peak was observed at Rf=0.38 which was exactly equal to that of a standard quercetin. As a result, the fact that this peak is aligned with the specific position and exhibits its strong magnitude give strong evidence that quercetin is a major constituent of flavonoid in the tuberous extract of Raphanus sativus.

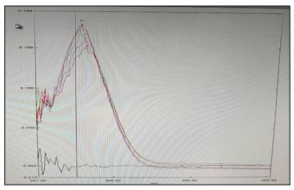
3.3.2 UV–Visible Spectroscopic Confirmation

The UV-Visible spectroscopic study was done to confirm the flavonoid nature and electronic attributes of the compound isolated in the tuberous part of the Raphanus sativus L. This technique proves especially illuminating with flavonoids, which have conjugated aromatic structures which give rise to the characteristic bands of absorption which can be used to classify the structure and preliminarily identify.

The spectral data of the isolated product were measured in the wavelength range of 200 to 600 nm in the ethanol and 'uv' was recorded. The spectrum revealed a strong absorption peak(λmax) at about 370-375nm, which is the spectrum of Band I. flavonol-type compounds. Band I is formed as a result of electronic transitions $\pi \rightarrow \pi$ which happen in the cinnamoyl system (B-ring conjugated with the C-ring carbonyl group) and is used to diagnose quercetin and flavonol of structural analogs.

There was also a weaker absorption band at the lower wavelength range (around 250-270nm) that was attributed to Band II, which is associated with the benzoyl system of the A- ring. The presence of both the Band I and Band II absorptions at the same time supports the presence of the canonical flavonol chromophore system, thus the identification of the isolated compound as quercetin. The λmax measured was very close to the UV spectral properties of quercetin standard analyzed under the same conditions and comparable values reported in the literature. The fact that no additional or displaced absorption bands were observed meant that the compound isolated was not a mixture of flavonoids, but a relatively pure flavonol fraction. Furthermore, the co-extracted impurities appeared to be very minimal since the absorption peak is sharp and symmetrical. Therefore, it can be concluded that the UV–Visible spectral behaviour of the obtained compound gives strong arguments to support the presence of quercetin. As it is coupled with chromatographic data and other spectroscopic tests, UV-Visible spectroscopy was a critical factor in identifying the identity of the isolated flavonoid as well as the success of the extraction and isolation approach used in this study.

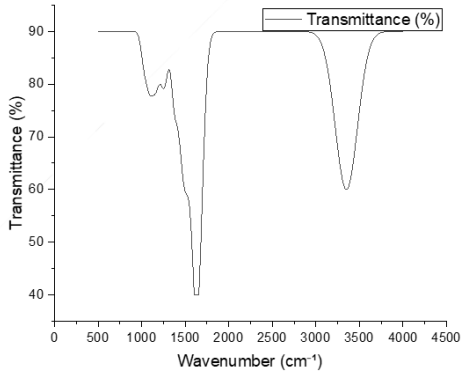




The UV-Visible spectrum of the obtained isolate showed a sharp absorption maximum (λmax) at the area around 370-375nm, which was a typical feature of flavonol-type constituents. This absorption band can be traced to the π→π* transition characteristic of the conjugated aromatic system and fits with the reported UV spectral characteristics of quercetin, thus supporting its identification.

3.3.3 FTIR spectroscopic confirmation

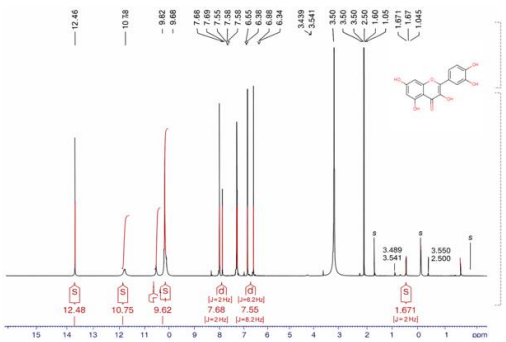
The measurement technique FTIR spectroscopy provided functional-group-level information on the isolated compound. The spectrum contained a broad absorption around 3390 cm^{-1} min and this can be attributed to hydrogen-bonded phenolic O H vibration. C=O flavonoid carbonyl group was identified as to have a peak value of around 1655 cm^{-1} because C=C aromatic modes were present at about 1605 and 1510 cm^{-1}. Other absorptions in these ranges 1260-1030 cm^{-1} were attributed to C-O and C -O-C vibrations. Collectively, the given spectral properties get in good agreement with the FTIR spectra already reported about quercetin.



The FTIR spectrum of the individual component showed that it had a broad band of phenolic OH stretching around 3390 cm^{-1}. The high absorption at 1655 cm^{-1} of the flavonoid skeleton was a good indication of C=O, whereas aromatic C=C vibrations were seen at 1605 cm^{-1} and 1510 cm^{-1}. C–O and C–O-C stretching modes were attributed to peaks in the 1260-1030 cm^{-1}. Such spectroscopic features are in strong agreement with the FTIR profile reported of quercetin, and therefore confirm the flavonoid nature of the obtained material.

3.3.4 ^{1}H-NMR spectroscopic confirmation

This confirmation was also done with the aid of the Structural confirmation by the use of the NMR spectroscopy which consists of the use of the protons in structures. The spectrum showed clear downfield singlets of protons on the phenolic hydroxyl group, a characteristic feature of quercetin and this is because of the existence of intra-molecular hydrogen bonding. The aromatic region showed well resolved proton signals that were in agreement with the A- and B-ring substitution pattern of quercetin. The chemical shifts, multiplicities and couple constants strongly matched literature published values, thus providing a strong support on the flavonol skeleton.



^1H-NMR Spectroscopy
^{1}H-NMR (400 MHz, DMSO-d_6, δ ppm): 12.48 (s, 1H, 5-OH), 10.75 (s, 1H, 7-OH), 9.62 (s, 1H, 3-OH), 7.68 (d, J≈2 Hz, 1H, H-2′), 7.55 (dd, J≈8,2 Hz, 1H, H-6′), 6.88 (d, J≈8 Hz, 1H, H-5′), 6.42 (d, J≈2 Hz, 1H, H-8), 6.19 (d, J≈2 Hz, 1H, H-6).

3.3.5 Overall confirmation

Taken together, the consistent chromatographic behaviour (TLC and HPTLC), characteristic UV–Visible absorption, functional group signatures in FTIR spectra, and detailed proton NMR patterns provide convincing confirmation that the isolated compound is quercetin. The agreement of results across multiple analytical platforms demonstrates the reliability of the extraction and isolation strategy and validates the identification of quercetin as a major flavonoid constituent of the Raphanus sativus tuber.

Taken altogether, the concordant chromatographic (TLC and HPTLC) behaviour, the typical UVVIS absorption, the presence of the functional-group characteristics in the FTIR spectra and the proton/NMR patterns all provide strong arguments that the compound obtained is quercetin. The similarity in the yield of the analysis on different analysis instruments serves to confirm the effectiveness of the extraction and isolation method hence the identification of quercetin as a leading flavonoid component of Raphanus sativus tuber.

3.6 Comparative Evaluation

Findings made in the current research are close to those reported in the literature on Raphanus sativus and other flavonoid-containing plant sources in the past. Previous research has always found radish tubers as a good source of flavonoids especially quercetin and polar solvents like ethanol have a better extraction capacity. This was evident in the current work in which the ethanol extract had a higher flavonoid content when compared to the ethyl acetate extract.

The UV-visible peak, which is at 370-375 nm, is similar to the reported UV-visible Band I absorption of quercetin in other previous studies. In the same manner, the bands in the FTIR functional groups analysis and the Rf value of TLC and HPTLC analysis are similar to those of quercetin in purified matrices of the radish and other related plants. This comparison is also supported by the 1H-NMR spectral data which demonstrated chemical shifts and proton patterns of quercetin.

On the whole, the similarity of the current results and the literature reports prove the utility of the extraction, isolation, and characterization procedures used and allows defining quercetin as a primary flavonoid compound of the Raphanus sativus tuber.

CONCLUSION

This research was able to extract, isolate, characterize and quantify quercetin in the tuberous portion of Raphanus sativus L. by using a sequence of solvent extraction and screened phytochemicals with ethanol, which was an effective solvent to extract the flavonoid. The chosen chromatographic method allowed quercetin to be isolated successfully since the TLC and HPTLC profiles were reproducible.

Extensive spectroscopic characterization by means of the use of UV -Visible, FTIR and 1H -NMR techniques gave unambiguous and mutually supportive evidence of identification of the isolated compound as quercetin. Quantitative analysis also found that quercetin is significant overall constituent to total flavonoid content of the radish tuber extract, with respect to the phytochemical richness of this edible portion of the plant. Comprehensively, the results confirm the tuberous portion of the plant, Raphanus sativus to be a useful and readily available source of quercetin in nature. The article also gives a simple and replicable methodological design which can be used with other comparable plant matrices. These findings suggest the possibility of using radish tubers in the production of nutraceutical and phytopharmaceutical products and has scientific grounds on the traditional and dietary value of radish tubers.

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CONFLICT OF INTEREST STATEMENT

The authors affirm that there are no conflicts of interest associated with this publication

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None.

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