International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 22-10-2021, Accepted: 07-11-2021, Published: 23-11-2021
Volume 6, Issue 6, 2021, Page No. 474-478

Phytochemical analysis and *In vitro* antibacterial activity of different solvents in root extract of *Raphanus sativus* L

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Abstract

In the present investication suggested that the phytochemical constituents and their functional and antibacterial properties of *Raphanus sativus* root extract were performed. The phytochemicals such as alkaloids, carbohydrate, coumarins, flavonoids, glycosides, phenols, protein, saponins, steroids, tannins, terpenoides, and quinones with different solvents of aqueous, methanol, hexane and diethyl ether were extracted and analysed from *Raphanus sativus*. The *Raphanus sativus* root extract of antibacterial properties of different concentration of 25, 50, 75 and 100µl was tested against bacteria such as *Bacillus* Sp, *E.coli*, *K.pneumoniae* , *Pseudomonas aeroginosa* and *Staphylococcus aureus* were determined from the aqueous and methanolic extracts of root extract of *Raphanus sativus* in 100µl concentration was better proformance when campared with low concentration. However, the test plant root extract was suitable candidature for antibacterial activities with specific concentration.

Keywords: *Raphanus sativus*, antibacterial activity, bacterial strains, phytochemical

Introduction

R.sativus belongs to family Brassicaceae. *R.sativus* is a food crop, mostly an ingredient of salads in Asian countries during winter. Its familiar names include black radish (English), Mooli (Urdu) and Daikon (Japanese). It has been used as a medicinal plant from a long time. It has laxative effects on intestine and acts as an appetizer (Chevallier, 1996) [7], used for curing liver dysfunction and poor digestion (Gutierrez and Perez, 2004; Lugasi *et al.*, 2005) [11, 17].

Medicinal plants have long been associated with the production of secondary metabolites which include tannins, terpenoides, coumarins, alkaloids and flavonoids. These products help plant to carry out various activities like defence and pollination. However, their antioxidant, antimicrobial and other medicinal properties are widely exploited for the benefit of mankind regarding healthcare. Certain biological assays are conducted in order to assess the phytochemicals and antimicrobial potentials of a plant (Cowan, 1999) [8].

Screening of plants extracts for antimicrobial activity has shown that Brassicaceae plants represent a potential source of anti-microbial compounds (Prasad *et al.*, 2015) [23]. Radish, *Raphanus sativus* L. var. longipinnatus of Brassicaceae family is widely grown all over the world. Extract of *R. sativus* is previously reported with a diverse range of metabolites with regard to biopharmaceuticals (Lugasi, 2005; Kim, 2011) [17, 16]. Aqueous extract of the whole plant is found potent against bacterial strains like *Sarcina lutea* and *Staphylococcus epidermidis* (Caceres, 1987) [5]. Parts of the plant like leaves, roots and seeds have also been analyzed individually for antibactiral activity and found active against many pathogens *Bacillus* sp, *E.coli*, *K.pneumoniae*, *Pseudomonas aeroginosa* and *Staphylococcus aureus* (Javaid and Bashir, 2015) [15]. Seeds of radish are rich source of chemical compounds like phenols, alkaloids, glycosides and sterols (Gutiérrez and Perez, 2004) [11]; these compounds are believed to potential against several pathogenic microbes (Candido, 2011) [6]. The main objectives of this study is assess non-volatile phytochemicals and antibacterial property of radish seed oil against selected bacterial species. (Nadia Khan *et al.*, 2019)

The search for new antimicrobial compounds has always been a need. The designed to dig out the antibacterial potential of waste material like peels of *R. sativus*. This preliminary study support the fact that like seeds (Rani *et al*., 2008) [24], roots (Esaki and Onozaki, 1982) [9] and leaves (Firas and Bayati, 2009) [10] of *R. sativus* peels also showed antimicrobial activity against some pathogenic gram positive and gram negative bacteria.

As compared to synthetic antimicrobial agents, plant based antimicrobials are cost effective, affordable and exhibit lesser side effects. The antibacterial potential of waste material like peels of *R. sativus*. *Raphanus sativus* contains raphanin compound responsible for its strong antifungal and antibacterial activity. Similarly, citrus genus belongs to the

large family *Rutaceae* were found to contain flavonoids, coumarins and cartenoids which play an important role in prevention of many degenerative diseases such as Alzheimer's and Parkinson's disease. **(**Sourav Rathour, *et al*.,2020) [28] The antibacterial activity of root extracts of the *R.sativas* obtained by serial extraction (using petroleum ether, chloroform, methanol, and water) and by *invitro* techniques and preliminary screening phytochemicals present in the extract by qualitative means standard methods against clinically isolated drug-resistant *E. coli* and *K. pneumoniae*; preliminary phytochemical screening was taken to detect the presence of alkaloids, saponins, flavonoids, steroids, tannins, reducing sugars, phenolics, protein, and oil and fat. Determination of antibacterial activity showed that the test organisms were susceptible to methanol and aqueous extracts only. MIC of methanolic extract was found to be 20 µg/mL on both *E. coli* and *K. pneumoniae* while aqueous extract had MIC of 10 and 20 µg/mL on *E. coli* and *K. pneumoniae* respectively. Preliminary phytochemical screening showed the presence of all the above-mentioned phytochemicals. (Asad Syed *et al*, 2020)

Materials and Methods

Collection of Rhizome of *Raphanus sativus* and triazole

Healthy *Raphanus sativus* seed was collected from Agricultural Department, Thanjavur, Tamil Nadu, India. Root of *R. sativus*, a common vegetable used as a salad in winters were collected from allowed to dry in shade for 2 weeks.

Pot-Control

Pot-Foliar spray of Hexaconazole @ 25mg/L

Pot-Foliar sprat of Uniconazole @ 25mg/L

Pot-Foliar sprat of Hexaconazole + Uniconazole + DAP @ 25:25:25 mg/L

Sample preparation

Dried peels were ground in an electric grinder to obtain fine powder. The powdered sample was stored in sterilized air tight container at room temperature (25-30°C). Extract preparation of *R. sativus* root sample were prepared using different types of organic solvents and water.

Qualitative Phytochemical Analysis (Harbone *et al.*, 1973)

Phytochemical test were carried out of the aqueous extract and methanol extract on the powdered specimens were using standard procedures to identify the constituents.It was done to assess the qualitative chemical composition of crude extracts using commonly employed, precipitation and colorations reaction to identify the major natural chemical groups such as alkaloids, carbohydrate, coumarins, flavonoids, glycosides, phenols, protein, saponins, steroids, tannins, terpenoides, and quinones. General reactions in these analysis revealed the presence or absence of these compounds in the plant extracts.

Test for alkaloids

One ml of HCL and Mayar's reagent (2ml of 5%) was added to 1ml of root extract of *Raphanus sativus* root. The formation of green precipitate indicated the presence of alkaloids.

Test for coumarins

Extract solution was concentrated to yield a residues. Dissolve residue in hot water. After cooling divide solution in two test tubes. To one test tube added 10% (w/v) Ammonium Hydroxide. Other test tube is used as control. Fluorescence color indicated the presence of coumarin.

Test for carbohydrates (Molisch's Test)

One ml of the Molishs reagent was added then along the walls of the test tube carefully conc H_2SO_4 was added. Formation of a brown ring at the junction of two liquids was observed.

Test for flavonoid

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color which becomes colorless on addition of dilute acid indicated the presence of flavonoids.

Test for glycosides (Borntrager's test):

To the extract solution (1 mL), 5% H_2SO_4 (1 mL) was added. The mixture was boiled in a water bath and then filtered. Filtrate was then shaken with equal volume of chloroform and kept to stand for 5 min. Then lower layer of chloroform was shaken with half of its volume with dilute ammonia. The formation of rose pink to red color of the ammoniacal layer gives indicated of anthraquinone glycosides

Test for phenols

Ferric chloride and few drops of ethanol was added to 1ml of root extract of *Raphanus sativus.* Formation of violet color indicated the presence of phenols.

Test for protein

Two ml of filtrate was treated with 2ml of 10% sodium hydroxide solution in a test tube and heated for 10 minutes. A drop of 7% copper sulphate solution was added in the above mixture. Formation of purplish violet colour indicate the presence of proteins.

Test for Saponins

Few drops of water and two drops of coconut oil were added to root extract of *Raphanus sativus* root formation of layer or foam, indicated the presence of saponins.

Test for steroids

Acetic acid (2ml) was added to 2ml of root extract of *Raphanus sativus* boiled then allowed to cool and added sulphuric acid the formation of upper green color layers was positive and presence of steroids.

Test for tannins

Five ml of the tepal extract was placed in a test tube and then 2 ml of 5 % of $FeCl_3$ solution was added. A greenish-black precipitate indicated the presence of tannins.

Test for terpenoids

Two milliliter of chloroform was mixed with the plant root extract and evaporated on the water bath then boiled with 2 ml of concentrated H_2SO_4. A grey color produced and indicated the entity of terpenoids.

Quantitative Phytochemical analysis

Estimation of Alkaloids (Harborne 1973)

One gram of the *Raphanus sativus* root was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and its covered and allowed to stand for 4 hrs. It was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated NH_4OH was added by drop wise to the extract until the precipitation was completed. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH_4OH and then filtered. The residue was the alkaloids which dried and weighed.

Estimation of Carbohydrates (Trease and Evens 1983):

Carbohydrates with free aldehyde or ketone groups reduce copper sulphate to cuprous oxide forming a yellow or brownish red coloured precipitate. Fehling's reagent is prepared freshly by mixing equal volumes of two stock solutions A and B. Solution A is 6.93 grams of $CuSO4.5H_2O$ per 100 ml of water and Solution B is 20 grams of KOH and 34.6 grams of sodium potassium tartarate (Rochelle salt) per 100 ml solution. Add a few drops of sugar solution at a time to 5 ml of Fehling's solution and heat the mixture after each addition. The production of yellow or brownish red cuprous oxide precipitate indicated the presence of reducing sugars.

Estimation of flavonoids: **(Bohm and Kocipai-Abyazan 1994)**

One grams of plant root sample was repeatedly extracted with 100ml of 80% aqueous methanol at room temperature. The mixture was filtered through a Whatmann No1 filter paper into a pre-weighed 250ml beaker. The filtrate was transferred into a water bath and allowed to evaporate to dryness and weighed.

Determination of gylcosides: **(Harborne 1973)**

Cardiac glycoside content in the sample was evaluated using Buljet's reagent as described by El-Olemy .One gm of the fine powder of *Raphanus sativus* was soaked in 10ml of 70% alcohol for 2hrs and then filtered. The extract obtained and then purified using lead acetate and Na_2HPO_4 solution before the addition of freshly prepared Buljet's reagent (containing 95ml aqueous picric acid +5ml 10% aqueous NaOH). The difference between the intensity of colours of the experimental and blank (distilled water and Buljet's reagent) samples gives the absorbance and is proportional to the concentration of the glycosides.

Estimation of protein: (Harborne 1973)

The total proteins content was determined by using Bradford's method.The 100 µl of the root sample extract added 3 ml of Bradford's reagent and incubated in dark for 5 minutes. The absorbance was measured at 595nm. Bovine serum albumin dilutions (0.1mg/ml to 0.5mg/ml) are used as standard solutions.

Estimation of saponins: (Obadoni and Ochuko 2001).

The rood samples were ground. 20g of each plant samples were dispersed in 200 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined root extracts were reduced to 40 ml over water bath at about 90°C. The concentration was transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight. The percentage of saponins content was calculated.

Estimation of tannins (Van – Burden and Robinson, 1981):

Five hundred mg of the *Raphanus sativus* root of 1gm sample was weighed into a 50 ml plastic bottle. 50ml of methanol and aqueous solvent was added and shaken for 1 hrs in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipette out into a test tube and mixed with 2 ml of 0.1M F_eCl_3 in 0.1 N HCL and 0.008 M potassium ferro cyanide. The absorbance was measured at 120 nm within 10 mm.

Estimation of terpenoids: (Harborne 1973):

Dried *Raphanus sativus* extract 100mg (wi) was taken and soaked in 9ml of methanol and aqueous for 24 hour. The extract after filtration, was extracted with 10mL of petroleum ether using separating funnel. The ether extract was separated in pre-weighed glass vials and waited for its complete drying (wf). Ether was evaporated and the yield (%) of total terpenoids contents was measured by the following formula (wi-wf/wi×100).

Determiantion of antibacterial activity (Perez *et al.*, 1990)

Preparation of *Raphanus sativus* extract

The aqueous and methanol extracts of *Raphanus sativus* root were prepared with different concentration of 25, 50, 75, and 100µl used used for antimicrobial activity by well diffusion method.

Test microorganisms

The following bacterial and fungal strains were used for the screening of antimicrobial activity. Bacteria, *Bacillus* sp, *E.coli*, *K.pneumoniae*, *Pseudomonas aeroginosa* and *Staphylococcus aureus* were selected for this study.

Media used

Nutrient agar (NA) were used for tested the antibacterial activity

ii) Agar well – diffusion method:

Agar well-diffusion method was followed for determination of antibacterial activity. Nutrient agar (NA)) plates were swabbed (sterile cotton swabs) with 24 hours culture and 48 hours old-broth culture of respective bacteria. Agar wells (5mm diameter) were made in each of these plates using sterile cork borer. About 25, 50, 75 and 100µL of aqueous and methanol extracts were added using sterilized dropping pipettes into the wells and plates were left for 1 hour to allowed a period of pre-incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions the plates were incubated

in an upright position at 37˚C ± 2˚C for 24 h for bacteria. Results were recorded as the presence or absence of inhibition zone. Triplicates were maintained and the average values recorded for antibacterial activity.

Results and discussion

In Indian folk medicine, *Raphanus sativus* and root used as a household remedy especially for the treatment or recovery for many diseases. The most familiar examples are jaundice, liver diseases, indigestion and other gastric pains.

In the present investigation the Phytochemical analysis and *invitro* antibacterial activity of different solvent in root extract of *Raphanus sativus* L.plant. The phytochemical screening (qualitative and quantitative) such as alkaloids, carbohydrate, glycosides, flavonoids, phenol, protein, saponin, steroids, tannin and terpenoids were represented in *Raphanus sativus* and these phytoconstituents were responsible for all the biological activity against bacteria and function of cellular mechanisms were performed.(Table-1,2) The effects of antibacterial properties were investigated. It was different concentration of 25, 50 75, and 100µl concentration of *Raphanus sativus* root extract with different solvents like aqueous and methanolic treated compound (1mm) were analysed. The zone of inhibition was measured with bacterial growth like *Bacillus* sp, *E. coli, K.pneumoniae, Pseudomonas aeroginosa* and *Staphylococcus aureus* were performed. The maximum zone of inhibition was observed from *Staphylococcus aureus* and minimum level from *Bacillus* sp, in aqueous extract of *Raphanus sativus* whereas methanolic extract also the same tread was observed because solvent has main role for the activity of phytocompounds. Effect of *R.sativus* with different concentration were treated against bacteria. (Table-3, 4)

The of *R. sativus* ethanol extract contained 145.91 ± 0.18 mg GAE/g phenolic and 21.95 ± 1.2 mg RE/g flavonoid compounds. (Heshu *et al* 2020) [14]. Phytochemical tests detected presence of many compounds including carbohydrates, amino acids alkaloids, flavonoids, and glycosides in radish seeds. (Nadia Khan *et al.,* 2019) Phytochemical analysis revealed the presence of tannins, saponins, flavonoids, phlobatannins, anthraquinones, carbohydrates, reducing sugars, steroids, phytosterol, alkaloids, amino acids, terpenoids, cardiac glycosides and chalcones in *R. sativus* niger extracts (Safia Janjua *et al*.,2013) (Table-1,2) The phytochemical compounds are carbohytrade, flavonoids and protein are present in the all solvents and coumarins phenols and steroids are absent of all solvents. Aqueous and methanol are will performed solvents. Antibacterial assay performed against pathogenic bacterial species showed that *Clavibacter* sp., and *E. coli* were susceptible to amoxicillin (positive control) by inhibiting approximately 18 mm in both bacterial species but resistant to radish seed oil. *Xanthomonas* bacterial sp. did not showed any results. Thus, it is concluded that either the used bacterial species are more resistant strains or the oil extract does not possess anti-pathogenic agent against tested bacterial species. (Nadia Khan *et al.*,2019). Zone of inhibition in mm .Extract concentration of 100 mg/ml is effective against both gram positive and gram negative bacterial strains tested. Ethyl acetate extract was most effective against *S. aureus, B. subtilis, S. typhi* and *K. pneumonia*. Ethanol extract had highest zone of inhibition against *M. luteus, P. aeruginosa, B. bronchisepticae and E. aerogenes.* (Safia Janjua *et al*.,2013). (Table-3, 4) The antibacterial activity of aqueous and methanol extract are zone inhibition result most effective against *Bacillus* sp, *E. coli, K.pneumoniae, Pseudomonas aeroginosa* and *Staphylococcus aureus*.

Table 1: The qualitative analysis of phytochemical compounds of *Raphanus sativus* root extract

Phytochemical compounds	Different solvents			
	Aqueous	Methanol	Hexane	Diethyl ether
Alkaloids	+	+	-	-
Carbohytrade	+	+	+	+
Coumarins	-	-	-	-
Flavonoids	+	+	+	+
Glycosides	-	-	+	-
Phenols	-	-	-	-
Protein	+	+	+	+
Saponins	-	+	-	-
Steroids	-	-	-	-
Tannin	+		-	-
Terpenoids	-	+	-	+

(+) present (-) absent

Table 2: The Quantitative analysis of phytochemical compounds of *Raphanus sativus* root extract

Phytochemical compounds	Quantity (mg/g)			
	Aqueous	Methanol	Hexane	Diethyl ether
Alkaloids	2.00±0.19	3.20±0.14	-	-
Carbohytrade	3.00±0.23	2.40±0.16	1.20±0.13	1.00±0.25
Flavonoids	4.00±0.21	3.00±0.20	1.02±0.15	1.00±0.22
Glycosides	-	-	1. 02±0.22	-
Protein	3.00±0.22	3.00±0.16	1. 03±0.24	1.00±0.24
Saponins	-	2.00±0.23	-	-
Tannin	2.00±0.24	-		-
Terpenoids	-	1.00±0.22	-	1.00±0.20

Values are expressed by mean ± S.D

Table 3: The antibacterial activity of *Raphanus sativus* root with aqueous extract against bacteria

Name of the bacteria	Zone of inhibition (mm)			
	25µl	50 µl	75 µl	100 µl
Bacillus sp.	02.0±0.14	04.2±0.11	04.2±2. 06	06.0±3.10
E. coli	05.0±0.13	11.0±0.09	10.0±2.10	15.0±2.06
K. pneumoniae	06.2±0.45	05.3±0.11	11.0±0.32	11.0±3.47
Pseudomonas aeroginosa	07.0±0.57	08.7±0.67	09.3±0.42	08.7±2.77
Staphylococcus aureus	13.0±0.13	12.3±0.11	11.0±2. 03	15.4±0.43

Values are expressed by mean ± S.D

Table 4: The antibacterial activity of *Raphanus sativus* root with methanol extract against bacteria

Name of the bacteria	Zone of inhibition (mm)			
	25µl	50 µl	75 µl	100 µl
Bacillus sp.	08. 0±0.11	07.0±0.37	10.0±0.47	11.2±2.28
E. coli	11.3±0.74	11.0±0.10	12.0±0.13	10.0±1.46
K. pneumoniae	14.4±0.55	13.3±0.24	11.0±0.20	12.5±0.84
Pseudomonas aeroginosa	10.6±0.83	15.0±0.10	09.0±0.14	11.4±0.50
Staphylococcus aureus	05.3±0.62	14. 0±0.25	14.0±0.12	13.2±0.43

Values are expressed by mean ± S.D

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