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Assessment of antioxidant and antimicrobial Activities, along with the quantification of diverse phytoconstituents using GC-MS And HPTLC of the methanolic extract of leaves of *Raphanus sativus*

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ABSTRACT

This study delves into the phytochemical composition and pharmacological potential of the methanolic extract derived from the leaves of *Raphanus sativus*. Preliminary phytochemical analysis revealed the presence of glycosides, flavonoids, cardiac glycosides, tri-terpinoids, and phytosterols. Thin Layer Chromatography (TLC) demonstrated the existence of Kamferol, Apigenin, Chlorogenic Acid, and Caffeic Acid in the extract. High-Performance Thin-Layer Chromatography (HPTLC) further quantified Kamferol (0.59 mcg/ml), Caffeic Acid (0.16 mcg/ml), and Chlorogenic Acid (2.29 mcg/ml). The amino acid profiling has shown the good food value of the extract and that has resulted with L-Glutamic acid (0.08 µg/ml), L-Histidine (0.053 µg/ml) and L-tyrosine (0.079 µg/ml). Gas Chromatography-Mass Spectroscopy (GC-MS) analysis identified 25 compounds, with 5-Hydroxymethylfurfural and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy exhibiting radical scavenging and anti-proliferative activities. Additionally, 7,10,13-Hexadecatrienoic acid, (Z,Z,Z), displayed anti-bacterial, anti-oxidant, immune-stimulant, and anti-tumor activities. Notably, n-Hexadecanoic acid, a major constituent (Area% - 12%), possesses anti-inflammatory properties.

Quantitative analysis indicated a total phenolic content of 55.57±0.37 mg/100g and flavonoid content (quercetin) of 115.73±0.21 mg/100g. The extract exhibited significant DPPH radical scavenging activity (IC50 = 20.0±0.1 µg/ml) compared to ascorbic acid (IC50 = 43.5 ±0.35 µg/ml). Evaluation of antimicrobial activity has shown against the gram-negative bacteria *E. coli* & *S. typhi* with zone of inhibition 8.5 mm and 7.2±0.1 mm respectively & compared with gram-positive *S. aureus* and *Bacillus subtilis* with zone of inhibition 6.8±0.1 mm and 7.1 mm at the lowest concentration of 50 mg/mL, supported by GC-MS analysis identifying potential antimicrobial compounds.

The presence of n-Hexadecanoic acid suggests potential anti-inflammatory effects, offering promise in mitigating disorders like diabetes. In-silico and in-vivo studies are recommended to further explore these effects. The study establishes R. sativus leaves as a rich source of diverse phyto-constituents with multi-directional health benefits, supporting its potential as a valuable nutritional supplement.

Keywords: Radish leaves, raphanus, antibacterial, phyto-chemistry, phytochemical analysis

Introduction

In our daily lives, consuming a variety of fruits and vegetables holds significant importance in enhancing human health and preventing various diseases. These foods are regarded as rich sources of essential vitamins such as C, A, B6, thiamine, niacin, and E, as well as valuable minerals and dietary fibres. Fresh fruit and vegetables prove the presence of the same. Among fresh fruit the radish is a biennial herbaceous plant, part of the Brassicaceae family. It is cultivated for its young and tender tuberous roots, which develop from both the primary root and hypocotyls. The radish is renowned for its commercial value and nutritional benefits. The leaves of radish (Raphanus sativus) are rich in a variety of phytochemicals, particularly flavonoids, which are known for their antioxidant, anti-inflammatory, anti-cancer, and anti-diabetic properties. These flavonoids play a crucial role in regulating processes like carbohydrate digestion, insulin signaling, insulin secretion, glucose uptake, and adipose deposition (Vinayagam R and Xu B, 2021). They target multiple molecules involved in key pathways, such as enhancing β-cell proliferation, promoting insulin secretion, reducing apoptosis, and improving hyperglycemia by regulating glucose metabolism in the liver. Examples of flavonoids found in radish fruits include flavonols like Quercetin, Kaemferol, Isorhamnetin, flavones like Apigenin, Luteolin, anthocyanins like Cyanidin, Pelargonidin, and isoflavonoids like Genistein (Manivannan A *et. al*., 2019).

A study conducted on diabetic rats induced with Streptozotocin (STZ) suggests that radish extract can inhibit GLUT2, consequently reducing glucose absorption in the small intestine (Vessal, M., Hemmati, M. and Vasei, M, 2003). Additionally, radish seeds contain Sulphorphene, a major isothiocyanate that has demonstrated anti-proliferative properties, potentially reducing cellular death caused by oxidative damage. Radishes are recognized as a significant source of vitamin C (ascorbic acid) and various minerals such as calcium and phosphorus (Lim, S *et.al*. 2020). Both the leaves and roots of Raphanus sativus have been historically used to treat cancer and address urinary complaints and piles (Malik MS *et. al*., 2010 & Kim J.W, Kim M.B. and Lim S.B., 2015). On the other hand, citrus fruits like lemon, orange, and grapefruit are excellent sources of vitamin C, folic acid, carotenoids, dietary fibres, potassium, selenium, and a wide range of phytochemicals. Vitamin C and citrus flavonoids are known for their potent antioxidant properties (Xi W. *et. al.,* 2014 & Manthey J. A., Grohmann K. and Guthrie N., 2001). Conducting a phytochemical evaluation of radish leaves will enable the identification and quantification of various biochemical compounds present in this plant part. Moreover, this study aims to demonstrate the antimicrobial and antioxidant properties along with the phytochemical & amino acid profiling of the methanolic extract obtained from the leaves of *Raphanus sativus*.

Materials & Methods

Plant material

The leaf part of the plant *Raphanus sativus (R. sativus)* were collected from Mursidabad district of the West Bengal. The plant was identified and authenticated by the survey of Botanical Survey of India, Howrah with the authentication number **JIS/SS-01.**

Preparation of Extract

The collected fresh leaves were shade dried, macerated with methanol for five days with continuous agitation and then the methanol was evaporated using rotary evaporator (40-45)°C and the final extract was collected.

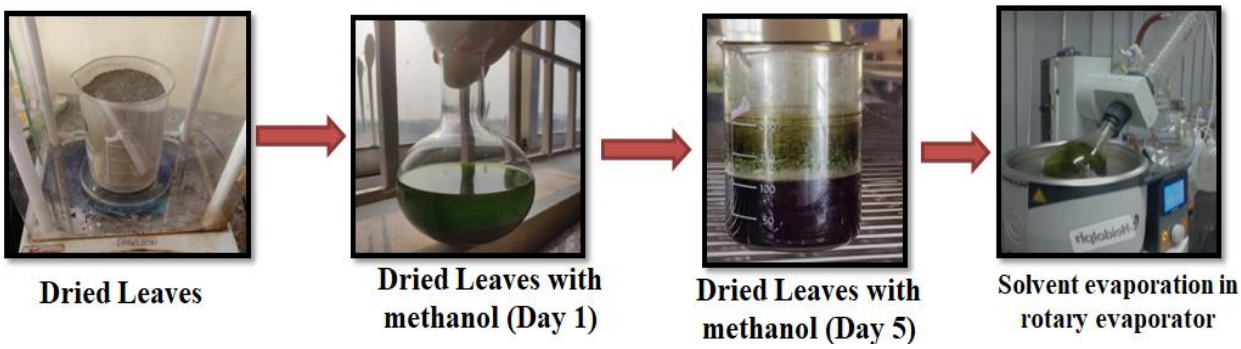


Fig 1: Extraction of *Raphanus sativus* leaves

Phytochemical Analysis

Phytochemical screening: Qualitative study of secondary metabolites

The qualitative phytochemical analysis of the *R. sativus extract* was carried according to Kokate et al, 2005 with slide changes (Kokate C.K., 2005).

Phytochemical screening: Thin Layer Chromatography (TLC)

Thin layer chromatography was used to analyse the extract in order to identify the various phenolic components that is present. Using formic acid (5:4:1v/v/v), ethylacetoactate, and chloroform as the mobile phase. Ultra violet (UV) and visible light were used to see the separated components, or visible spots. The migratory behaviour of the separated compounds (visible spots), express as a retardation factor (Rf value), was used to assess the plates qualitatively (Fougère L *et. al.,* 2019).

Phytochemical screening: Phenolic compounds profiling by HPTLC

With slide modification of Ashraf et al, 2021 the quantitative analysis of the plant extract (*R. sativus*) was performed using HPTLC method by Vision Cat software analysis. Plant extract (*R.sativus*) was applied on HPTLC pre-coated silica gel G F_{254} TLC plate plates (Merck K GaA, Supelco,Germany) compared with numerous standard phenolic (Ashraf G.J. *et. al.,* 2021) using the automatic TLC Sampler (ATS 4, CAMAG, Muttenz, Switzerland). Here used 100 µl of HPTLC syringe (Hamilton, Bondauz, Switzerland) and chloroform: ethyl acetate: formic acid (5:4:1 v/v/v) and toluene: methanol (9:1 v/v) as mobile phase. The spot's migration was then observed under TLC-UV Cabinet 4 after drying. The evaluation of chromatograms was done at shorter (254nm), longer wavelength (366nm) and visible light (Agatonovic-Kustrin, S. and Morton, D.W., 2017)

Phytochemical screening: Amino acid profiling by HPTLC

Using a CAMAG applicator, extracted samples and standard amino acid mixes were deposited as 1x6 mm bands on an HPTLC aluminum plate measuring 20 x 10 cm (Silica gel 60 F_{254} Merck). Prior to applying the sample, the plates were activated at 100 °C for 20 minutes. For the final application, each individual standard amino acid was sprayed onto an HPTLC plate and separated into five groups. Millipore water was used to dissolve individual amino acids at a concentration of 100 parts per million. Tryptophan was dissolved in 0.05 N NaOH, whereas phenylalanine and tyrosine were dissolved in 0.05 N HCl (Giji S. *et. al.,* 2011)

Phytochemical screening: GC-MS Analysis

The methanolic extract of *R. sativus* was subjected to GC-MS analysis for identification of volatile compounds. The GC-MS analysis was done in the Central University of Punjab using instrument

Shimadzu QP 2010 Ultra GC -MS. Helium is used as carrier gas with at low of 1.0 ml/min. The identification of components was based on NIST libraries as well as comparison of their retention time (N. U. Olivia, U. C. Goodness and O. M. Obinna, 2021). The constituents were identified after comparison with those available in the computer library (NIST) attached to the GC-MS instrument and the results obtained have been tabulated.

Invitro Antioxidant Assay

Total phenolic content

Phenolic compounds have redox properties, which allow them to act as antioxidants as their free radical scavenging ability is facilitated by their hydroxyl group (-OH). The study was carried out by the following procedure with small modification of the process Rad, S.K. and Movafagh, A, (2021). The TPC were determined from the linear equation (Y = 0.003x + 0.0306) of a standard curve prepared with Gallic acid and the amount of total phenolic content in the extract was expressed in milligram per 100 gram of extract.

Total flavonoid content

Determination of total flavonoid content was carried out by the following procedure with small modification of the process Ghasemzadeh *et al.,* (2010). The TFC was determined from the linear equation (Y = 0.003x + 0.0306) of a standard curve prepared with quercetin and the amount of total flavonoid content in the extract was expressed in milligram per gram of extract.

DPPH radicle Scavenging Activity

DPPH scavenging activity was measure according to Chatterjee et al, 2019 with some changes. UV-VIS spectrophotometer (Shimazu UV -1780) was used to observe absorbance at 517 (Xie J. and Schaich, K.M., 2014). The antioxidant activity was determined by this equation:

$$\text{DPPH scavenging activity (\%)} = (A_{Control} - A_{test}) / A_{Control} * 100$$

Where, $A_{Control}$: The absorbance of the control reaction

A_{test}: The absorbance in the presence of the sample of *R. sativus*

Hydrogen Peroxide (H2O2) radical scavenging activity

Method described by Ruch et al (1989) was utilized to measure the scavenging activity of *R. sativus* against H_2O_2 . A 40 mM solution of H_2O_2 was mixed with different concentrations of *R. sativus* solution. Absorbance was determined after 10 min at 230 nm. Phosphate buffer was considered as blank and ascorbic acid were treated as reference antioxidants.

$$\%\ H_2O_2 \text{ radical Scavenging activity} = (A_{Control} - A_{test}) / A_{Control} * 100$$

Where, $A_{Control}$: The absorbance of the control reaction

A_{test}: The absorbance in the presence of the sample of *R. sativus*

Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of *R. sativus* was done based on the method described by Liu et al. (1997) and Ye et al. (2000) with slight modification. Sample (*R. sativus)* was mixed with nitro-blue tetrazolium (NBT) solution (156 mmol/L NBT) and nicotinamide adenine dinucleotide (NADH) solution (468 mmol/L). 100 ml of phenazine methosulfate solution (60 mmol/L) was mixed to the above solution to start the reaction. The incubation temperature was 25°C for 5 min, and the absorbance was measured at 560 nm. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

% inhibition=($A_{Control}$-A_{test})/$A_{Control}$ *100

Where, $A_{Control}$: The absorbance of the control reaction

A_{test}: The absorbance in the presence of the sample of *R. sativus*

ABTS scavenging activity

ABTS scavenging activity was performed by the slide modification of Chatterjee et al, 2019 using ABTS salt, potassium persulfate at 734 nm. The ABTS scavenging capacity of *R. sativus* was compared with ascorbic acid and the percentage inhibition calculated as follows:

ABTS radical scavenging activity (%) = ($A_{Control}$-A_{test})/$A_{Control}$ *100

Where , $A_{Control}$: The absorbance of the control reaction

A_{test}: The absorbance in the presence of the sample of *R. sativus*

Antimicrobial Study

R. sativus extract were further assessed for antibacterial activity against gram-negative bacteria like *E. coli* (ATCC-10231), *S. typhi* (NCTC-786) and gram- positive bacteria *S. aureus* (ATCC-7953), *Bacillus subtilis* (ATCC-9144) through disk diffusion method (Parvekar P *et. al.,* 2020). Freshly prepared cultures media, Mueller Hinton agar were inoculated with different microbial strain by using sterile paper disk (0.6 mm diameter). Paper disk prepared with different concentration of *R. sativus* solution (50 mg/ml, 100mg/ml, and 200mg/ml). Incubation of the plates was done at 37◦C for 24-48 h.

Statistical analysis

The data were analysed by one-way ANOVA followed by Dennett's t-test GraphPad Prism and GraphPad InStat software. The data were expressed as mean ± SD. The significance was considered when $p<0.05$.

Result

Phytochemical screening: Qualitative study of secondary metabolites

The preliminary phytochemical study of the extract of *R. sativus* revealed the presence or absence of various secondary metabolite (Table1). The study has shown the presence of glycoside, flavonoids, cardiac glycoside, triterpinoids, phytosterols in the methanolic extract of *R. sativus.*

Table 1: Identification of various secondary metabolites

Sl. No.	Secondary Metabolites	Result
1.	Glycosides	+
2.	Flavonoids	+
3.	Cardiac glycosides	+
3.	Saponin Glycoside	-
4.	Tri-Terpenoid	+
5.	Tannin	-
6.	Alkaloids	-
7.	Phytosterols	+

[(+) means positive result (-) means negative result]

Phytochemical screening: Thin Layer Chromatography (TLC)

The result of TLC study has shown that the extract of *R. Sativus* leaves has majorly four phyto-constituents (polyphenol) predominantly among the twelve different standards. P-Cumaric acid, Gallic acid, T-Cinnamic acid, Vanillic acid, 4-Hydroxybenzoic acid, Kaempferol, Quercetin, Apigenin, Naringenin, Rutin, Chlorogenic acid and Caffeic acid were used. The spot's migration was then observed under TLC UV cabinet-4 at UV 254 nm, UV 366 nm light, and Visible Light 466 nm (Figure 2).

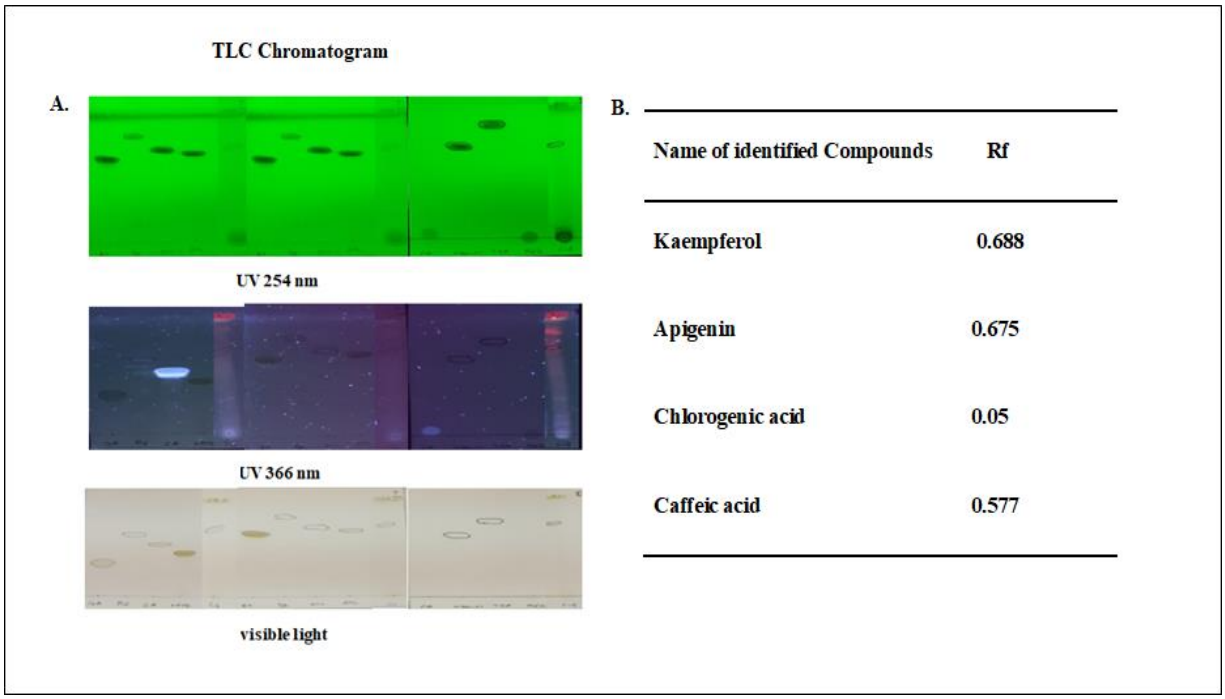
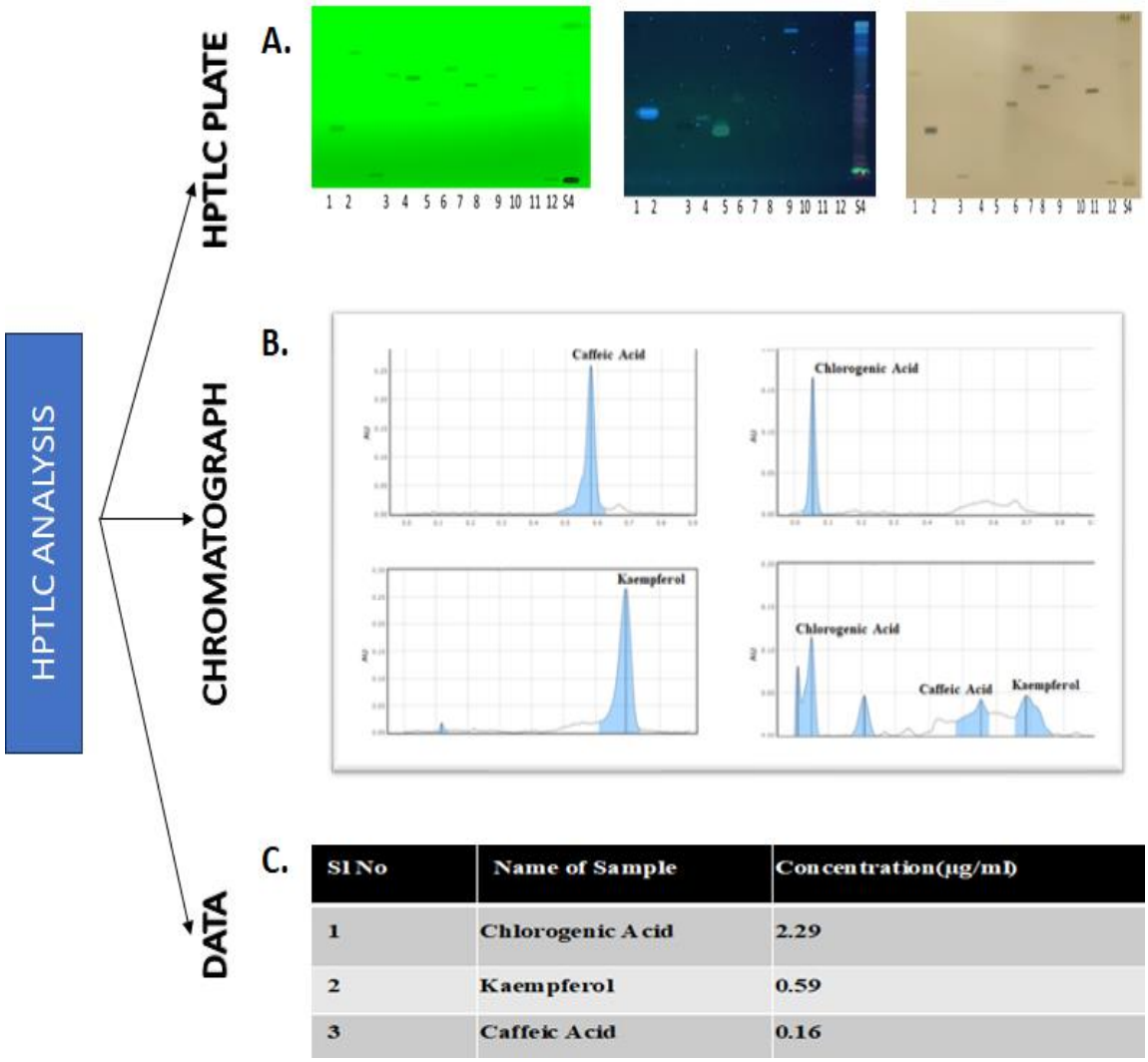


Figure 2 A: TLC Chromatogram of the extract of *Raphanus sativus* at UV 254, UV366, & Visible Light. 2B List of phenolic compounds in Raphanus sativus extract identified in TLC chromatogram with their respective retention factor (Rf).

High Performance Liquid Chromatography (HPTLC)

12 different standards like P-Cumaric acid, Gallic acid, T-Cinnamic acid, Vanillic acid, 4-Hydroxybenzoic acid, Kaempferol, Quercetin, Apigenin, Naringenin, Rutin, Chlorogenic Acid and Caffeic acid were used. From methanolic extract of *R.sativus,* kaemferol, caffeic acid, and chlorogenic acid was identified and quantified 0.59 µg/ml, 0.16 µg/ml, and 2.29 µg/ml, respectively, using the HPTLC analysis (Figure 3).

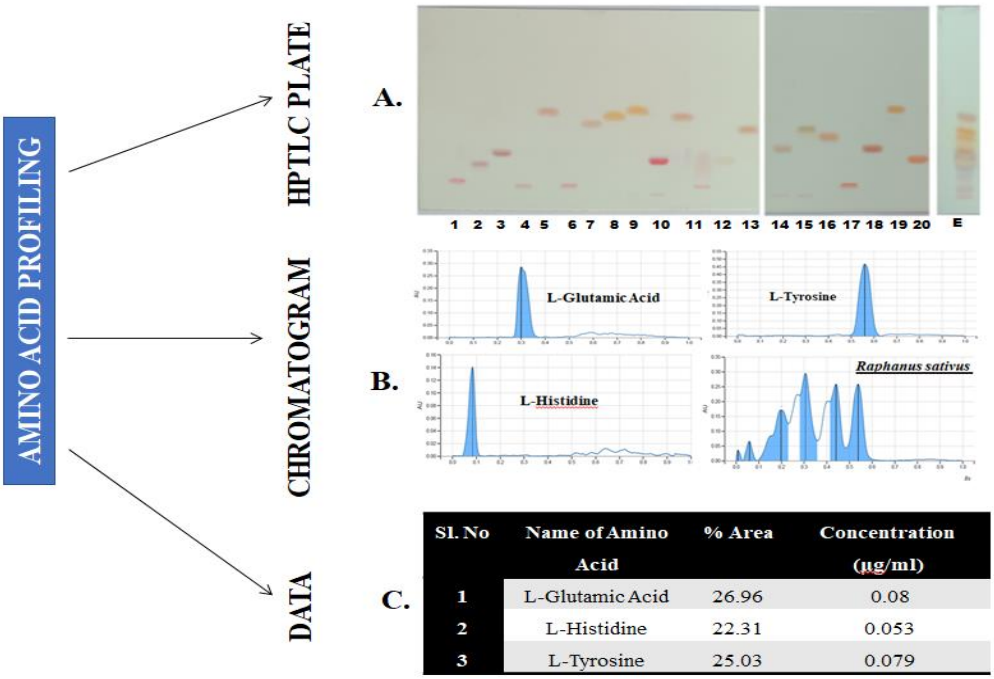


Sl No	Name of Sample	Concentration(µg/ml)
1	Chlorogenic Acid	2.29
2	Kaempferol	0.59
3	Caffeic Acid	0.16

Figure 3 A: HPTLC fingerprinting for extract *R. sativus* under visible light, UV 254nm and UV 366nm. Mobile phase Chloroform : Ethyl acetoacetate : Formic Acid at a ratio 5:4:1v/v. Track 1 to 13 with standard compounds are p- coumaric acid ; gallic acid ; trans cinnamic acid ; chlorogenic acid; vanillic acid ; 4-hydroxy benzoic acid ; myricetin ; quercetin; apigenin ; naringenin ; caffeic acid ; rutin and extract respectively. Figure 3B: HPTLC Chromatogram of the standard flavonoids, phenolic compound and extract. Figure 3C: Quantification of identified compounds.

Phytochemical screening: Amino acid profiling by HPTLC

The amino acid profiling was done using 20 various amino acids like L-Arginine monohydrochloride, DL-Aspartic Acid, L-Glutamic Acid, L-Histidine monohydrochloride, L-Leucine, L-Lysine monohydrochloride, DL-Methionine, L-Tyrosine, DL-Phenylalanine, DL-Serine, DL-Isoleucine, L-Cysteine monohydrochloride, L-Proline, DL-Valine, L-Hydroxyproline, DL-Threonine, 3-(3,4-Dihydroxyphenyl)-DL-alanine [DL-DOPA], DL-2-Amino-n-butyric acid, L-Ornithine monohydrochloride, DL-Alanine. Among these amino acids, the leaf extract of *Raphanus sativus* contains L-glutamic acid (0.08 µg/ml), L-Histidine (0.053µg/ml) and L-tyrosine (0.079 µg/ml)



C.

Sl. No	Name of Amino Acid	% Area	Concentration (µg/ml)
1	L-Glutamic Acid	26.96	0.08
2	L-Histidine	22.31	0.053
3	L-Tyrosine	25.03	0.079

Figure 4 A: HPTLC fingerprinting of extract *R. sativus* under visible light, UV 254nm and UV 366nm. Mobile phase Chloroform : Ethyl acetoacetate : Formic Acid at a ratio 5:4:1v/v. Track 1 to 21 with standard compounds are L-Arginine monohydrochloride, DL-Aspartic Acid, L-Glutamic Acid, L-Histidine monohydrochloride, L-Leucine, L-Lysine monohydrochloride, DL-Methionine, L-Tyrosine, DL-Phenylalanine, DL-Serine, DL-Isoleucine, L-Cysteine monohydrochloride, L-Proline, DL-Valine, L-Hydroxyproline, DL-Threonine, 3-(3,4-Dihydroxyphenyl)-DL-alanine [DL-DOPA], DL-2-Amino-n-butyric acid, L-Ornithine monohydrochloride, DL-Alanine and extract respectively. Figure 4B: HPTLC Chromatogram of the standard amino acid and extract. Figure 4C: Quantification of identified compounds.

Phytochemical screening: GC-MS Analysis

GC-MS analysis of the methanolic extract of *R. sativus* was done to identify and quantify the various volatile compounds present. The active principles with their retention time (RT), molecular formula, concentration (peak area %) and previously identified biological effects are presented in Table 3 which shows the presence of 25 bioactive phytochemical compounds in the methanolic extract of *C. R. sativus*. The compounds which have higher %area, have been highlighted in the table as having higher in concentration in the extract may claim to provide most of the prominent biological benefits. The mass spectra of identified compounds of *R. sativus* are given in Figure 5.

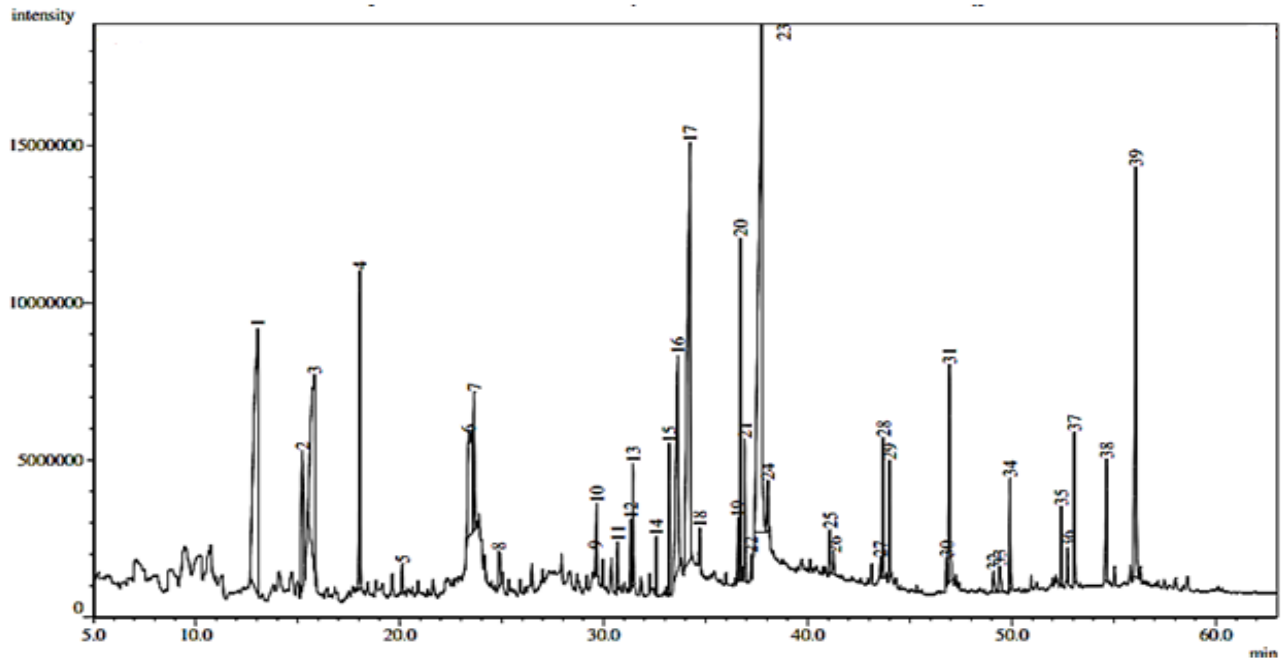


Figure 5: GC-MS chromatogram of methanolic extract of *R. sativus*

Table 3: Phytochemical constituents present in the methanolic extract o *R. sativus*

Sl No	Name	Chemical Formula	RT Time	% Area	Biological effects	Reference
1	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy	C6H8O4	13.043	11.60	Anti-oxidant activity	(Chen Z. *et. al.,* 2021)
2	4-Vinylphenol	C8H8O	15.220	2.98	Anti-angiogenic and anti-tumor effect	(Yue G. G *et. al.,* 2015)
3	5-Hydroxymethylfurfural	C6H6O3	15.820	7.46	Anti-oxidant and anti-proliferative activity	(Zhao. L *et. al.*, 2013)
4	2-Methoxy-4-vinylphenol	C9H10O2	18.053	3.99	Anti-microbial,anti-inflammatory and anti-oxidant activity	(Rubab M. *et. al.,* 2020)
5	L-Proline, 5-oxo-, methyl ester	C7H11NO3	20.130	0.40	Antioxidant and Antiinflammatory activity	(Amala V and Jeyaraj M., 2014)
6	1[5'(Hydroxymethyl)furfuryl] pyrrolidine	C10H15NO	23.375	4.55	Selective Inhibitor of DNA Polymerase	(Mizushina Y. *et. al.,* 2006)

7	**Phenol, 4-ethenyl-2,6-dimethoxy-**	C10H14O3	24.849	0.71	Antioxidant and COX-2 Inhibitor	(Rubab *et. al.,*2020)
8	**Tetradecanoic acid**	C14H28O2	29.664	0.68	Antioxidant, anticancer preventive, Nematicide, Lubricant, Hypocholesterolemic	(Juárez-Rodríguez MM *et. al.,* 2021)
9	**1H-Indole-3-acetonitrile**	$C_{10}H_8N_2$	30.686	0.50	Anti-influenza	(Zhao X. et. al., 2021)
10	**Neophytadiene**	C20H38	31.321	0.62	Antioxidant antibacterial activities	(Rautela Indra *et. al.,* 2020)
11	**2-Pentadecanone, 6,10,14-trimethyl-**	C18H36O	31.426	1.09	Anti-Bacterial	(Yue X. *et. al.,* 2017)
12	**9,12,15-Octadecatrienoic acid**	C18H30O2	32.571	0.51	Antioxidant, Antimicrobial, and Anti-inflammatory activities	(Siswadi, S. and Saragih, G. S., 2021)
13	**Hexadecanoic acid, methyl ester**	C17H34O2	33.194	1.28	Antibacterial, antioxidant,antitumor, immunostimulant	(Rautela Indra *et. al.,* 2020)
14	**7,10,13-Hexadecatrienoic acid, (Z,Z,Z)**	C16H26O2	33.632	5.69	Antibacterial, antioxidant,antitumor, immunostimulant	(Rautela Indra *et. al.,* 2020)
15	**n-Hexadecanoic acid**	C16H32O	34.216	12.00	Anti-Inflammatory activity	(Aparna V. *et. al.,* 2012)
16	**9H-Pyrido[3,4-b]indole**	C11H8N2	34.698	0.35	Antifilarial chemotherapy	(Srivastava S. K. *et. al,* 1999)
17	**9,12-Octadecadienoic acid (Z,Z)-, methyl ester**	C18H32O2	36.571	0.58	Anti-inflammatory, hypocholesterolemic, hepatoprotective	(Krishnamoorthy, K. and Subramaniam, P

., 2014)

18	**Phytol**	C20H40O	36.909	1.42	Anticancer, antioxidant, and antimicrobial	(Islam M. T. *et. al.,* 2020)
19	**9,12,15-Octadecatrienoic acid, (Z,Z,Z)**	C18H30O2	37.730	17.79	Anti-oxidant activity	(Tian C. *et. al.*, 2018)
20	**Octadecanoic acid**	C18H36O2	38.033	0.56	Antioxidant, anticancer, antimicrobial activities	(Mazumder K. *et. al.* 2020)
21	**Behenic alcohol**	C22H46O	43.699	1.28	Antimicrobial	(Ranganathan D., 2014)
22	**alpha.-Tocospiro A**	C29H50O4	49.110	0.33	Anti-tubercular activity	(Chen J. *et. al.,* 2010)
23	**Vitamin E**	C29H50O2	53.063	1.52	Antioxidant	(Rautela Indra *et. al.,* 2020)
24	**Campesterol**	C28H48O	54.627	1.75	Cholesterol lowering and anti-carcinogenic effects	(Choi J. *et. al.,* 2007)
25	**Gamma sitosterol**	C29H52O2	56.073	6.38	Antioxidant, free radical scavenging, anti-diabetic activity	(Rautela Indra *et. al.,* 2020)

In vitro antioxidant assay

Total Phenolic Content & Total Flavonoid Content

Total phenolic content and total flavonoid content of the plant can be correlated with the standard compounds like gallic acid and quercetin respectively. Here total phenolic content and total flavonoids content were estimated **115.73±0.21mg /100gm** and **55.57±0.37/100gm** equivalent to gallic acid and quercetin respectively in Table 4.

Table 4. Total Phenolic and flavonoid content of the extract of the plant *R. sativus*

Sl No	Experiments	Result (mg/100g)
1	Total Phenolic content	**55.57±0.37**
2	Total flavonoid content	**115.73±0.21**

DPPH Radicle Scavenging Activity

The DPPH radicle scavenging activity of the extract of the leaves of *R. sativus* as well as the reference standard ascorbic acid was shown in the Figure 6A. IC_{50} value was measured by

comparing the absorbance of different concentration of test sample with the standard curve of Ascorbic acid. The IC_{50} value of methanolic extract of *R. sativus* is 20.0±0.1 µg/ml compared to IC_{50} value of ascorbic acid 43.5 ±0.35 µg/ml (Table: 4).

Determination of hydrogen peroxide (H_2O_2) radical scavenging activity

The ability of the *R. sativus* extract to scavenge hydrogen peroxide was determined according to the method of Ruch et al. The scavenging ability of *R. sativus extract* on hydrogen peroxide is shown in Figure. 6B and compared with ascorbic acid as standards. The *R. sativus extract* was capable of scavenging hydrogen peroxide in an amount dependent manner. Figure 6B shows that the *R. sativus extract* had strong hydrogen peroxide scavenging activity with IC_{50}144.5±0.45 µg/ml (Table: 5B).

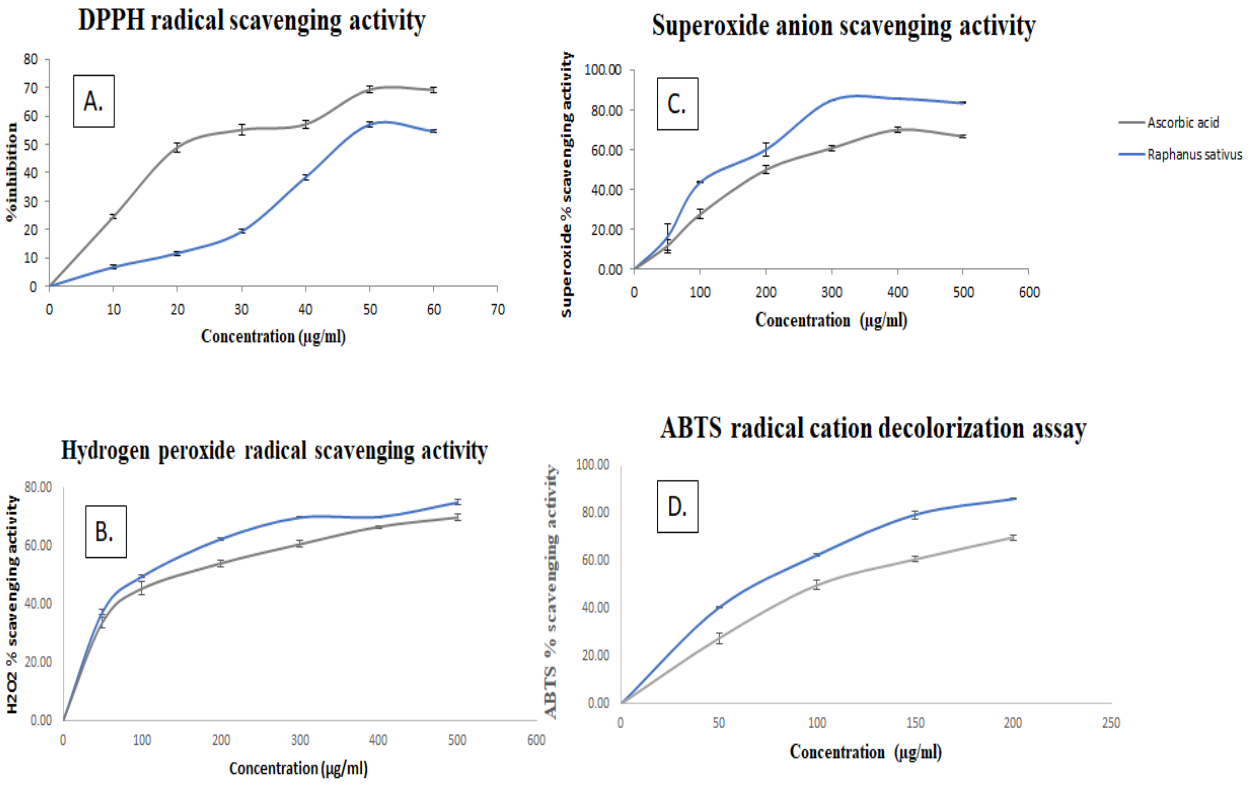


Figure 6: Antioxidant activity of the *R. sativus* extract at various concentrations. (A) DPPH radical scavenging activity, (B) H_2O_2 radical scavenging activity, (C) Superoxide radical scavenging activity, (D) 2, 2′-azinobis-3- ethylbenzothiazoline-6-sulfonic acid radical scavenging activity. The data were expressed as mean ± SD, n=3. The significance was considered when $p<0.05$

Superoxide anion scavenging activity

In the NADH-NBT system, NBT is reduced by the superoxide anion that is produced from dissolved oxygen through the NADH coupling mechanism. Thus, the consumption of superoxide anion in the reaction mixture is indicated by the decrease in absorbance at 560 nm with antioxidants. The % suppression of superoxide radical formation of 50-500 mg of *R. sativus* (IC50 140.1±0.1 µg/ml) extract is shown in Figure 6C, along with a comparison with the same doses of Ascorbic Acid (IC50 198.5±0.51 µg/ml) (Table 4).

ABTS scavenging activity

Figure 6D illustrates how *R. sativus* extract scavenges ABTS radicals in a concentration-dependent manner (25-250 µg/ml) in comparison to Ascorbic Acid. The outcomes demonstrated that the *R. sativus* extract outperformed Ascorbic Acid in terms of ABTS radical scavenging activity and possessed superior ABTS radical scavenging ability. Figure 6D shown IC_{50} values of *R. sativus* extract and Ascorbic Acid 65.6±0.34 µg/ml and 100.1±0.32 µg/ml respectively.

Table 4: Effect of scavenging activities of *R. sativus extract* on different Radical at different concentrations

Plant Extract/ Standard	IC_{50} Value (µg/ml) of different oxidant radicals			
	DPPH radical	H_2O_2 radical	Superoxide radical	ABTS radical
Ascorbic acid	43.5±0.35	100.0±0.41	198.5±0.51	100.1±0.32
R. sativus extract	20.0±0.1	144.5±0.45	140.1±0.1	65.6±0.34

Antimicrobial Study

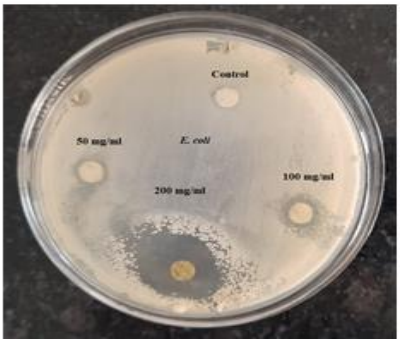
The zone of inhibition was calculated for determining the lowest concentration of antibacterial agent which would prevent the visible growth of a microorganism. The zone of inhibition of various concentrations of *R. sativus extract* is shown in Figure 7 A. The gram-negative bacteria *E. coli* (ATCC-10231) & *S. typhi* (NCTC-786) showed zone of inhibition 8.5 mm and 7.2±0.1 mm respectively & compared with gram-positive *S. aureus* (ATCC-7953) and *Bacillus subtilis* (ATCC-9144) which showed a zone of inhibition 6.8±0.1 mm and 7.1 mm at the lowest concentration of 50 mg/mL (Figure 7A, 7B, 7C). Zone of inhibition increased with the increasing concentration of extract. The *R. sativus extract* shown greater antimicrobial activity on gram-negative bacteria compared with the gram-positive bacteria.

Figure 7: Evaluation of antimicrobial activity of the *R. sativus* extract against pair of gram negative and gram positive bacteria using Zone of inhibition technique. Antibacterial activity of *R. sativus extract* by disk diffusion method: the pathogenic strains *E. coli* (B), and *Staphylococcus aureus* (C)

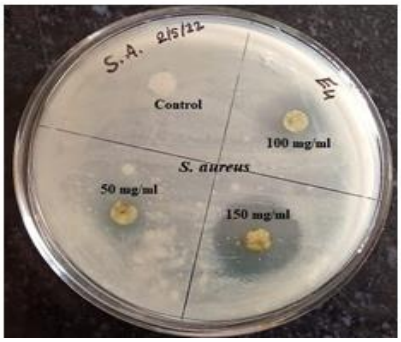
A.

Sl No.	Concentration (mg/ml)	Zone of Inhibition (mm)			
		Bacilus subtilis (ATCC-7953)	*Staphylococcus aureus* (ATCC-9144)	*S typhi* (NCTC-786)	*E. coli* (ATCC-10231)
1	50	7.10	6.80±0.1	8.50	7.20±0.1
2	100	7.17±0.06	6.50	8.53±0.15	7.30
3	200	7.20	8.00	8.53±0.06	7.60

B.



C.

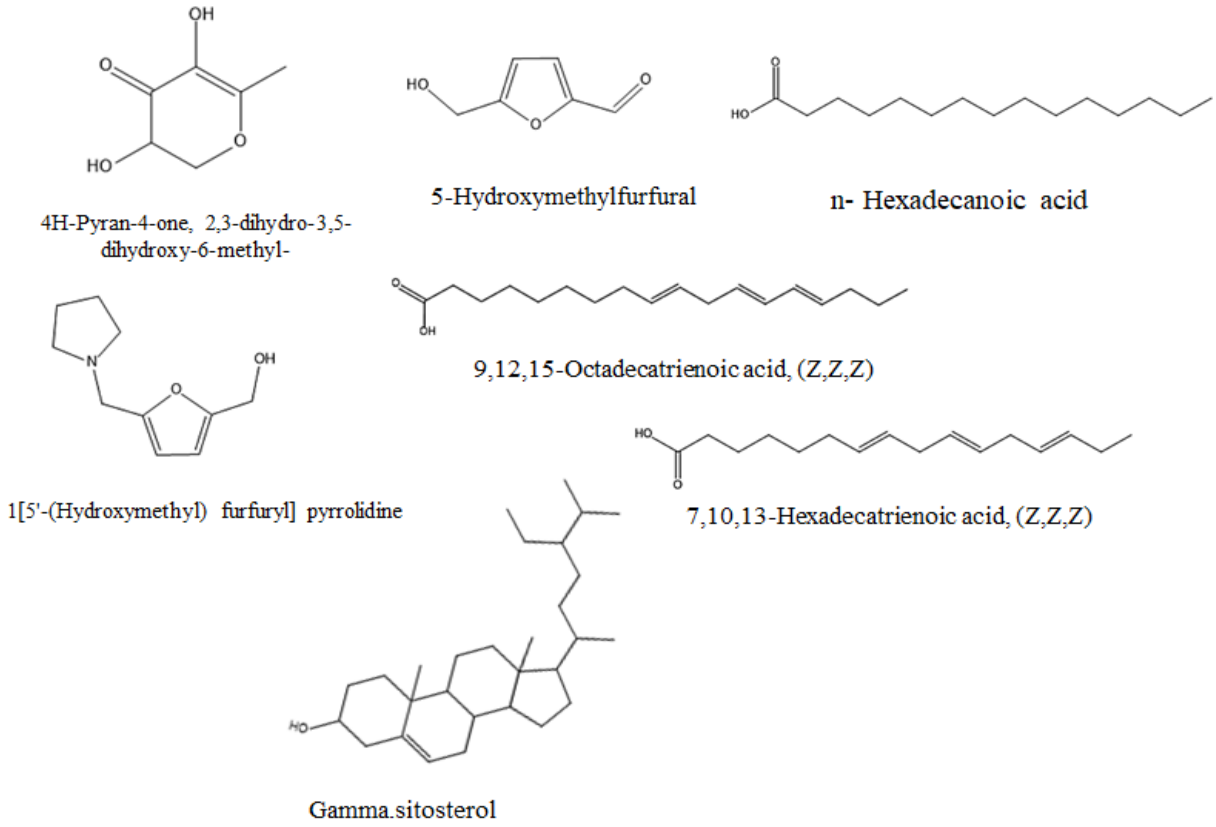


Discussion & Conclusion

The above-mentioned studies on methanolic extract of the leaves of *R. sativus* have proved various potential health benefits of the plant part. The phytochemical composition of R. *sativus* shows major constituents like flavonoids, non-flavonoids polyphenols, fat, etc which can be an integral part of healthy and balanced diet. The present study shows in the preliminary phytochemical analysis that *R. sativus* extract has glycoside, flavonoids, cardiac glycoside, tri-terpinoid and phytosterol (Table 1) which is genuinely a promising content to be considered for a nutritional supplement. Having flavonoids can be a proof of having antioxidant property in the plant [40]. The analysis of thin Layer chromatography (TLC) was done on *R. sativus* extract while 12 standard flavonoids (P-Cumaric acid, Gallic acid, T-Cinnamic acid, Vanillic acid, 4-Hydroxybenzoic acid, Kaempferol, Quercetin, Apigenin, Naringenin, Rutin, Chlorogenic Acid and Caffeic acid) used as standard compounds. TLC result reveal that *R. sativus* extract contains Kamferol (Rf -0.688), Apigenin (Rf 0.675), Chlorogenic acid (Rf -0.05) and Caffeic Acid (Rf- 0.577) in Figure 2A & 2B. Containing polyphenolic compounds makes promising substance to show anti-oxidant, anti-inflamatory and even anti-cancer property (Basli A., Belkacem N., and Amrani I., 2017). HPTLC study has been performed using different standard compound. The HPTLC result shown in Figure 4 by qualitative and quantitative analysis. Figure 4 showed the presence of three flavonoids as in Kamferol (0.59 mcg/ml), Caffeic Acid (0.16 mcg/ml) and Chlorogenic Acid (2.29 mcg/ml). As per the quantification Chlorogenic acid is mostly present in the extract which may confirm the anti-oxidant, anti-diabetic, anti-inflammatory, anti-carcinogenic effect (Tajik N. *et. al.,* 2017). The amino acid profiling of the extract by HPTLC method hass shown the presence of three essential amino acids which are L-Histidine, L-tyrosine and L-Glutamic acid [Figure 4]. Glutamic acid is an essential amino acid that plays a crucial role in neurotransmission and brain function. It is known for its potential to enhance cognitive function and memory. The amino acid L-Histidine is involved in the formation of histamine, a neurotransmitter that regulates various physiological processes, including immune response and inflammation. L-Tyrosine is a precursor to neurotransmitters like dopamine, norepinephrine, and epinephrine. These neurotransmitters are vital for mood regulation and stress response. L-Tyrosine supplementation has been linked to improved cognitive function, stress resilience, and mood stabilization (Ling ZN *et. al*., 2023). The Gas Chromatography-Mass Spectroscopy (GC-MS) study has been performed. A wide range of phytoconstituents in the extract which has actually helped to understand and demonstrate its potential pharmacological effects. By GC-MS analysis, twenty-five compounds have been identified (Figure 5 & Table 3) from *R. sativus* extract. Among all of these identified compounds, five compounds have significant quantity according to their maximum %area. According to chen et al, (2021), the existence of 5-Hydroxymethylfurfural and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy in the extract shows the radicle scavenging activity of the plant part (Chen Z. *et. al.,* 2021 & Zhao. L *et. al*., 2013) along with it has some anti-proliferative activity which can be beneficial for inhibition of cancerous cell growth (Zhao. L *et. al*., 2013). Mizushina et al, (2006) shown 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy has DNA polymerase inhibitor which is currently a great target for cancer treatment as DNA polymerase actually helps cancer cells to manage or cope up the DNA damage by anti-cancer drug (Mizushina Y. *et. al.,* 2006). The compound 7,10,13-Hexadecatrienoic acid, (Z,Z,Z) has strong anti-bacterial, anti-oxidant, immune-stimulant and also an anti-tumor activity Rautela Indra *et. al.,* 2020). Leaves extract of *R. sativus* has proved to have n-hexadecanoic acid as one of the major constituents in GC-MS analysis (Area% - 12%) which is an anti-inflammatory substance, can be extremely useful in various chronic disorders like Diabetes, Arthritis, Alzheimer's disease etc as progression of these diseases are closely related to inflammation (Aparna V. *et. al.,* 2012).

9,12,15-Octadecatrienoic acid, (Z,Z,Z) and Gamma- sitosterol is also present in a good amount in the extract where both has very significant anti-oxidant property which is very important for any type of degenerative disorders and the later has proved itself to reduce the high blood sugar level thus serves anti-diabetic characteristic (Rautela Indra *et. al.,* 2020 & Tian C. *et. al*., 2018).

Figure 8: Few chemical structures of major Phyto-constituents found in extract of the leaves of *R. sativus* by GC-MS analysis



The total phenolic content shows upright amount of phenolics in the extract which is 55.57±0.37 mg/100gm. There is presence of high amount of flavonoids in terms of quercetin is 115.73±0.21 mg/100gm. The study of DPPH radical scavenging activity proves the efficacy of the test compound that how much it's effective against free radicals or what quantity of that particular compound inhibits/stops the oxidation which leads to the various detrimental pathways of cellular damage which has been already proved by the presence of volatile constituents like 9,12,15-Octadecatrienoic acid, (Z,Z,Z), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy etc. in GC-MS analysis. The IC_{50} value of *R. sativus* extract against DPPH induced radicle scavenging activity is 20.0±0.1 µg/ml compared to IC_{50} value of ascorbic acid 43.5 ±0.35 µg/ml (Table: 4). IC_{50} value of *R. sativus* extract against H_2O_2 radicals, super oxide radicals and ABTS radicals are 144.5±0.45 µg/ml, 140.1±0.1 µg/ml, 65.6±0.34 µg/ml respectively as compare with Ascorbic acid as standard.

As it has already been seen that, the extract of *R. sativus* has some flavonoids which may confirms its antimicrobial activities along with the presence of Hexadecanoic acid, methyl ester, 2-Pentadecanone, 6,10,14-trimethyl- etc (Rautela Indra *et. al.,* 2020 & Yue X. *et. al.,* 2017) in GC-MS analysis justify the anti-microbial property of the plant extract. Flavonoids have proved itself to show inhibitory effects against various pathogenic microorganism. Increasing the antibiotic

resistance day by day has attracted flavonoids as a substitute of antibiotics (Xie Y. *et. al.,* 2014). The *R. sativus* extract has been shown antimicrobial activity against microorganism but it has greater antimicrobial activity on gram-negative bacteria compared with the gram-positive bacteria which proved by zone of inhibition.

As previously mentioned, one of the majorly available phyto-constituent found through GC-MS analysis was n-Hexadecanoic acid which is a potent anti-inflammatory compound which acts as a PLA_2 (Phospholipase A_2) inhibitor, catalyzes the hydrolysis of membrane glycerol-phospholipids to generate arachidonic acid, a precursor of prostaglandins and leukotrienes (Aparna V. *et. al.,* 2012). Prostaglandins and leukotrienes are the important factors to initiate the inflammatory response in the cell thus inhibiting the PLA_2 would be an important factor to reduce or prevent the inflammation. In the present world, diabetes is a disorder which can be an initiating factor for various diseases like cardiovascular disorders, renal failure etc. It has been seen that in type-II diabetes, increasing blood sugar level causes an elevated level of ROS (Reactive oxygen species) generation in the cell, which activates few pro-inflammatory proteins like Nf-kB, IL-6, IL-1 which as a response increases apoptotic proteins like caspase-3, caspase-9. Thus, activating caspase-9 indicates towards intrinsic pathway of apoptosis. Activation of caspase-3 ensures the cell death in diabetes (Khanra R. *et. al.*, 2015). Thus n-hexadecanoic acid in the leaves of Raphanus sativus can be useful to prevent the inflammation induced apoptotic cell death in type-II diabetes. This information can be well proved by the help of in silico study as well as the in-vivo study. From above discussion, it is proved that *R. sativus* extract have been full of potent phyto-constituents potential. So, it is an edible and easily available vegetable which show multi-directional activities in various health benefits.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of competing interest

The researchers claim no conflict of interests.

Credit authorship contribution statement

Sakshar Saha, Shubham Paul, Ritu Khanra: designed the experiments, supervised and participated entire work. Atanu Chatterjee: contributed to the writing of this manuscript. Rajarshi Jana, Arya Bhoumik, Milon Jana, Srijan Panigrahi, Dipanjan Sengupta, Triasha Mondal, Sreya Das: performed antimicrobial study and HPTLC study.

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