Original Research Article

Qualitative and quantitative phytochemical screening, antioxidant and anti-inflammatory activities of acetone extract of *Brassica juncea* L. leaf

ABSTRACT

OBJECTIVE: To evaluate the qualitative and quantitative phytochemical screening, antioxidant and anti-inflammatory activity of acetone extract of *Brassica juncea*(L.) Czern.~~L.(*B.juncea*)~~leaf.

METHODS: Qualitative and quantitative phytochemical screening were performed byfollowing standard protocols. Aluminum chloride method forquantification of the total flavonoidcontent, 3, 5-dinitrosalicylic acid (DNS) method for measuring glucose concentration, Folin-Ciocalteu reagent (FC reagent) for total phenol content and Lowry method for protein concentration respectively were used calorimetrically. To assess the antioxidant properties of acetone extract of *B.~~rassica~~juncea*~~L.~~ leaf, Nitric oxide (NO) assay, ~~,~~1-Diphenyl-2-picrylhydrazyl (DPPH), and 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid, ABTS)were used respectively. Molybdenum method was used to determine the total antioxidant activity of acetone extract of *B~~rassica~~.juncea*~~L.~~ leaf. Protease inhibitor assay and potassium ferricyanide methods were used to evaluate the anti-inflammatory and reducing power (%) activities of acetone extract of *B.~~rassica~~juncea*~~L.~~ leaf respectively.

RESULTS: Qualitative investigation of phytochemical constituents in acetone extracts of~~*Brassica*~~*B. juncea*~~L.~~ leaf showed the presence of alkaloids,carbohydrates,phenols, flavonoids,

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[3] . Sevindik M. Investigation of Antioxidant/Oxidant Status and Antimicrobial Activities of Lentinus tigrinus. Advances in pharmacological sciences. 2018. https://doi.org/10.1155/2018/1718025

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proteins,saponins and tannins respectively and absence of glycosides. Quantitative analysis of phytochemical constituents (biomolecules) in acetone extractsof ~~*Brassica*~~*B. juncea*~~L.~~ leaf showed flavonoid content (5.64 ± 00.09 µg/mg extract,quercetin used as standard), glucoseconcentration (97.74 ± 08.95 µg/mg extract,N-acetyl glucosamine used as a standard), phenolic content (120.15 ± 15.58 µg/mg extract,gallicacid used as a standard), protein content (398.42 ± 25.15 µg/mg extract, bovine serum albumin used as a standard) and total antioxidant activity (95.26 ± 07.85,ascorbic acid used as a standard) respectively. The inhibitory concentration (IC50) and EC50 values ofacetone extractsof ~~*Brassica*~~*B. juncea* L. leaf [(785.25 ± 15.85µg/mL (0.001 ± 0.001µg/mL), 615.29 ± 12.54 µg/mL (0.0016 ± 0.001µg/mL) and 975.85 ± 23.12µg/mL (0.001 ± 0.001µg/mL)] along with standards (gallic acid, quercetin and ascorbic acid) were estimated by using NO, DPPH and ABTS respectively. Protease inhibitor (%) and reducing power (%) activities were increased inacetone extractsof ~~*Brassica*~~*B. juncea* L. leaf dose dependent manner, at higher dose 1000µg/mL the Protease inhibitor (%) and reducing power (%) activities 537.95 ± 26.58 (%) and 41.28 ± 7.28 were observed respectively.

CONCLUSION: Based on our results, we concluded that acetone extracts of ~~*Brassica*~~ *B juncea* L. leaf showed potential antioxidant and anti-inflammatory properties but further research is required to known the compound which has potential antioxidant and anti-inflammatory properties and purification and separation of those bioactive compounds due to the efficiency of those bioactive compounds may increase when they are in pure form.

Keywords: *Brassica juncea*~~L.~~ leaf; Acetone extract; Phytochemicals; Antioxidants; Anti-inflammatory

INTRODUCTION

All organisms including human has a great defense systems such as antioxidant enzymes (glutathione peroxidase, superoxide dismutase, and catalase) and non-enzymatic antioxidants (glutathione, vitamins E and C, thiol antioxidants, melatonin, and carotenoids) against oxidative stress and numerous diseases but these defense systems cannot fully avert oxidative stress related damages.[1-4]

Oxidative stress is a main factor to cause various diseases (cardiovascular disease, rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, neurodegenerative and autoimmune diseases, and some cancers) due to production of reactive oxygen species or free radicals which leads to inflammation. These inflammatory processes can be prevented by antioxidant defense systems such as antioxidant enzymes (glutathione peroxidase, superoxide dismutase, and catalase) and non-enzymatic antioxidants (glutathione, vitamins E and C, thiol antioxidants, melatonin, and carotenoids). [5-8]

In market, there are numerous synthetic antioxidants available but it was reported in previous studies, those synthetic antioxidants are causing severe side effects.[9]The medicinal value of any medicinal plant depends upon the phytochemicals (alkaloids, flavanoids, tannins, phenolic compounds, anthocyanins, carotenoids, dietary glutathione, vitamins, and endogenous metabolites) present in it, in turn causes several physiological action in human body without any side effects.[10, 11]

So, the present study is depending on the plant secondary compounds which are involved in reducing oxidative damage should be used as alternative therapeutic drugs.[9] ~~*Brassica* *B.* *juncea* L. (*B. juncea*)~~ is economically important plant due to its uses (an oil seed, a green vegetable) and a medicinal plant to treat diabetic cataract[12], anti-inflammatory antinociceptive, anti-hyperglycemic activity, [13]backache, arthritis, paralysis, styes, edema of the lungs and liver, aperient, stimulant and emmenagogue[14]and antioxidant activity[15]etc. In China and Korea, *B. juncea* seed uses for arthritis, foot ache, lumbago, tumor, bladder infections, inflammation or haemorrhage and rheumatism. It is traditionally known as Indian mustard, Chinese mustard, oriental mustard, leaf mustard, or mustard green (Family- Brassicaceae).[16]It is grown throughout the world especially in India, china and other South Asia countries. It was earlier

reported that *B. juncea*having glycosides, flavonoids, phenols, sterols, triterpene, alocohols, proteins and carbohydrates.[17-20]

Hence, the aim of the present study is to investigate the qualitative and quantitative phytochemical screening, antioxidant and invitro anti-inflammatory activities of acetone extract of ~~*Brassica* *B.*~~*juncea*~~L. (*B. juncea*),~~ a medicinal plant.

MATERIALS AND METHODS

Sampling

The ~~*Brassica* *B.*~~*juncea*~~L.~~ herb was obtained from and maintained in a green house at Department of Horticulture and aromatic medicinal plants, Mizoram University, Aizawl, Mizoram, India. The samples were identified by Professor Rama Chandra Laha, a botanist in the Department of Botany, Mizoram University, Aizawl, Mizoram, India. The Voucher specimens (Ref. no. 531 were kept at herbarium, Department of Botany, Mizoram University, Mizoram, India.

Processing of sample and preparation of acetone extract

Young and tender leaves of~~*Brassica* *B.*~~*juncea*L. herb (Figure 1) were procured freshly and washed with distilled water thoroughly to remove dust particles. After processing, the fresh leaves were shed dried for 15 days in room temperature to make suitable for grinding then ground into a fine powder by using hand grinder (Prestige mixer grinder, Item code: 41407, kaiser appliances, Himachal Pradesh, India) and stored in amber colour tight bottles (Tarsons products Pvt. Ltd, Code: 581250, Kolkata, India) at 4 ◻ for further analysis. To make raw (crude) extract, 100 g (one hundred gram) of fine powder was added to 250 mL of absolute acetone (Acetone pure (99.5%), Catalog Number (15168), Sisco Research Laboratories Pvt. Ltd.Maharashtra, India) in 500mL of clonical flask (Catalog Number: 5021024, Borosil Glass Works Limited, Maharashtra, India) with constant and occasional shaking and stirring in orbital shaker (Catalog No: M1190-0002, New Brunswick™ Innova® 2000, 220/230 V/50/60 Hz, Eppendorf India Limited Headquarters, Ambattur, Chennai, Tamilnadu)at 300 rpm for 2 days. The homogenate was allowed to stand for 12 h and then filtered (by using No. 1 Whatman filter paper, Catalog No. 1001-020) and remaining acetone was evaporated (by using vacuum condensiate, Eppendorf 022820109 Vacufuge®

Plus Vacuum concentrator,Eppendorf India Limited Headquarters, Ambattur, Chennai, Tamilnadu) and was stored at 4 ◻ for further analysis. [21, 22]

Chemicals used for this study

Phenylbutazone 1, 1- diphenyl- 2- picryl- hydrazyl (DPPH), 2, 2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), trypsin, ascorbic acid, quercetin, galic acid were purchased from Sisco Research Laboratories Pvt. Ltd.(Maharashtra, India). Other all chemicals were used in this work were analytical grade (AR) procured from Merck & Co (New Jersey, United States).

Qualitative and quantitative phytochemical analysis

Qualitative analysis

The secondary (2°ry) metabolites of acetone extract of ~~*Brassica* *B*~~ *juncea*~~L.~~ herb leaf were analysed qualitatively by following protocols with slight changes. [23-25]

Alkaloids detection test

To treat extract, 2% of 1.~~5ml~~ 5mL~~HCl~~ HCLsolution was used and added. After HCL treatment, 0.5 mL of test sample aliquots was treated with 2 mL of Mayer's reagent (K_2HgI_4) and then observed yellow colour precipitate formation specified that presence of alkaloids.

Carbohydrates detection test

Carbohydrates were detected in extract sample by using molisch's test. In this test, 1 mL of molisch's reagent (α-naphthol dissolved in ethanol**)** was added to 1 mL of extract solution further conc. H_2SO_4 was added carefully along the walls of the test tube and then observed the light brown colour ring shape between the two liquid phases which inturn specified that presence of carbohydrates in the test sample (extract).

Glycosides detection test

Legal test was used to detect glycosides (cardiac glycosides) in the test samples (extracts), treated with sodium niropruside in pyridine and sodium hydroxide. After treatment, pink to blood red colour was developed, that denotes occurrence of glycosides (cardiac glycosides) in the test sample (extract).

Phenols detection test

Phenols were detected based on the development of bluish black colour in test reaction inferric chloride test, in which, 3-4 drops of 0.1% ferric chloride solution was added to 5 mL of test sample (extract).

Tannins detection test

Test sample (extract, 1mL) was boiled in 2 ml mLof Distilled water (D.W) and then filtered by using a No. 1 Whatman filter paper. After filtration, the filtrate was treated with a few drops of 0.1% ferric chloride solution. After treatment, either brownish-green or blue-black colour was developed and considered as a proof of the occurrence of tannins.

Flavonoids detection test

Alkaline reagent test was performed to detect the flavonoids in test samples (extracts). In which, few drops of sodium hydroxide (NaOH) solution was added to the 0.5 mL of the extract sample, After addition, intense yellow colour was developed and later vanished on the addition of dilute acids, indicates the occurrence of flavonoids.

Proteins detection test

Proteins were detected in extracts by following method namely, xanthoproteic test method, in which, few drops of conc. Nitric acid to be used to treat extracts. Later, yellow colour was developed and indicated that occurrence of proteins.

Saponins detection test

Forth test was used to detect saponins in a given test sample (extract). In which, extract (0.5 mL) was diluted in 5 mL of D.W. and then shaked vigorously 15 minutes. After shaking, 1 cm thick foam was developed inside the reaction tube, which evidenced that occurrence of saponins.

Quantitative analysis

Flavonoids, glucose, phenols, protein and antioxidant activities were estimated or quantified in acetone extracts of *Brassica B.juncea*L. leaf by using following standard methods with slight modifications. All experiments were repeated five times (n=5).

Flavonoid quantification method

Aluminum chloride method was used to quantify the total flavonoid content in acetone extract of *Brassica B.juncea*L. leaf, in which, 0.3 mL aluminum chloride (10%, w/v) and 0.3 mL potassium acetate (1 M) were added to the reaction tube contains acetone extract (0.5mL,100 µg/mL) was mixed with 2 mL of acetone to dilute the extract solution and then stands at room temperature for half an hour (30 minutes), followed by O.D. (absorbance) values were taken by using UV-Visible spectrophotometer (Eppendorf

BioSpectrometer ® basic, 230 V/50 – 60 Hz, Catalog No. 6135000009, Eppendorf India Limited Headquarters, Ambattur, Chennai, Tamilnadu) at the 415 nm wavelength. The graph was made with quercetin (as a standard) at a concentration (5-30 µg/mL) in 2 mL of acetone, followed by the resultant expression of total flavonoids is micrograms of quercetin equivalents per milligram of fresh extract (µg QE/mg extract). [25-28]

Glucose quantification method

Glucose was measured by using 3, 5-dinitrosalicylic acid (DNS) method. Briefly, in this method, 5 mL of DNS and 2 mL of 20% (w/v) sodium potassium tartrate were added to acetone extract (0.5mL, 100 µg/mL) in reaction tube followed by water bath incubation at 90°C for 10 minutes (red-brown colour) and then stand at room temperature for cooling for 15 minutes followed by O.D. measurements at 575 nm. The graph was made with N-acetyl glucosamine (as a standard) at a concentration (5-30 µg/mL) in 2 mL of acetone, followed by the resultant expression of total glucose is micrograms of N-acetyl glucosamine equivalents per milligram of fresh extract (µg GlcNAcE/mg extract). [28, 29]

Phenols quantification method

Folin-Ciocalteu reagent (FC reagent) was used to estimate the total phenols in acetone extract of ~~*Brassica* *B.*~~*juncea*~~L.~~ leaf. 1 mL of FC reagent was diluted in 15 mL of D.W., further 5 mL of diluted FC reagent was mixed with acetone extract (0.5mL, 100 µg/mL) was mixed with 2 mL of acetone to dilute the extract solution and then stand for 7 minutes at room temperature. After 7 minutes incubation, sodium carbonate (Na_2CO_3, 4 mL, 1M) was added and then allowed to stand at room temperature for 10 minutes for determine the colour reaction. Further, the O.D. values were taken at 765 nm wavelength by using UV-VIS spectrometer. Gallic acid (as a standard, 2-10 µg/mL in acetone) was used to make a standard graph. Based on standard graph, the phenols were determined in micrograms of gallic acid equivalents per milligram of fresh extract (µg GAE/mg extract). [25,26,28,30,31]

Protein quantification method

Proteins in acetone extract of ~~*Brassica* *B.*~~*juncea*~~L.~~ leaf were quantified by Lowry method. This method depends on two phases of reactions, 1) formation of Biuret chromophore complex 2) FC reagent reduction. In this method, the lowry's reagents were added to acetone extract (0.5mL, 100 µg/mL) was mixed with 2 mL of acetone to dilute the extract solution and then stand for 10 minutes at room

temperature for development of blue colour and further measured the O.D. values at 720 nm. Bovine serum albumin (BSA, as a standard, 5-30 µg/mL in acetone) was used to make a standard graph. Based on standard graph, the total proteins were determined in micrograms of bovine serum albumin equivalents per milligram of fresh extract (µg BSAE/mg extract). [28, 31, 32]

Total antioxidant quantification method

Total antioxidant capacity was measured in acetone extract of ~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf depending on the reduction in molybdenum states (Mo, VI – V) and emergence of coloured complex (green phosphate/Mo (V) complex) at acidic pH. 0.5 mL (100 µg/mL) of acetone extracts of ~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf was added to 5 mL of reagent solution in a reaction tube and then kept at 90 ˚C in water bath for 1 hour and 45 minutes. Further, the reaction container allowed to cool at room temperature for 15 minutes and then took the O.D. values at 695 nm. Ascorbic acid (AA, as a standard, 5-30 µg/mL in acetone) was used to make a standard graph. Based on standard graph, the total antioxidants capacity was determined in micrograms of ascorbic acid equivalents per milligram of fresh extract (µg AAE/mg extract).[33, 34]

Invitro studies

Antioxidant studies

Nitric oxide (NO) free radical scavenging assay (activity)

NO free radical scavenging activity of acetone extract of ~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf was performed using NO assay.Various concentrations of acetone extracts of ~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf were taken (10µg/mL, 50µg/mL, 250µg/mL, 500µg/mL and 1000µg/mL). 5 mL of each concentration, extract was mixed with 2ml of sodium nitroprusside solution (5mM) in a reaction vessel and then stands for 90 minutes at 30 ˚C. Further, a small amount (2.5 mL) of mixer solution was taken out by using pipette and mixed with 2.5 mL of Griess reagent and allowed for incubation for 5 minutes at room temperature and then taken O.D values at 550nm in UV-VIS spectrometer. Quercetin, Gallic acid and Ascorbic acid used as a positive control biomolecules. All experiments were repeated five times (n=5). [34, 35]The following formula determines the free radical scavenging activity.

$$\text{Free radical scavenging activity (\%)} = [(A_1-A_2)/A_1] \times 100$$

Where A_1 is the O.D. value of the NO solution without any test sample, A_2 is the O.D. value of the NO solution with any test sample (extracts and positive controls). The quantity or amount or concentration of

antioxidants in extracts of any living material is necessity to remove 50% of free radicals from the living system is called inhibitory concentration (IC50). All experiments were repeated five times (n=5).

1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (activity)

DPPH scavenging activity of acetone extracts of ~~*Brassica* *B*~~ *juncea*~~L.~~ leaf was measured using DPPH assay, in which, either hydrogen donating or radical scavenging activity was happened. Briefly, 2 mL of various concentrations (10 – 1000 µg/mL) of acetone extracts of ~~*Brassica* *B*~~ *juncea*~~L.~~ leaf was added to DPPH solution (2 mL, 0.12 mM) in a test tube and then allowed to incubation for 40 minutes after vigorous shaking in the dark conditions. After 30 minutes incubation, the O.D. values were measured at 530 nm. Acetone (negative control), quercetin, gallic acid and ascorbic acid used as a positive controls. The following formula determines the free radical scavenging activity.

$$\text{Free radical scavenging activity (\%)} = [(A_1-A_2)/A_1] \times 100$$

Where A_1 is the O.D. value of the DPPH solution without any test sample, A_2 is the O.D. value of the DPPH solution with any test sample (extracts and positive controls). The quantity or amount or concentration of antioxidants in extracts of any living material is necessity to remove 50% of free radicals from the living system is called inhibitory concentration (IC50). All experiments were repeated five times (n=5).[2~~5~~4, 2~~7~~8, 3~~3~~4, 3~~5~~6]

2, 2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) free radical scavenging assay (activity)

The working solution of ABTS was prepared (by mixing 100 µL of 100 mM potassium persulfate to 5 mL of ABTS) from the stock solution of ABTS (5 mM) and then stabilized and stored for 10-15 h in a dark condition at an ambient temperature. The 0.1mL of acetone extracts of ~~*Brassica* *B*~~ *juncea*~~L.~~ leaf (at various concentrations ranging from 10 – 1000 µg/mL) was added to the 1 mL of the ABTS amples in a test tube and allowed to heating at 37 ˚C for 5 minutes and then the O.D. values were observed in UV-VIS spectrometer at 720 nm. Only acetone sample without any other sample was considered as a negative (-) control where as Quercetin, Gallic acid and L-ascorbic acid were considered as a positive (+) control. The following formula determines the free radical scavenging activity.

$$\text{Free radical scavenging activity (\%)} = [(A_1-A_2)/A_1] \times 100$$

Where A_1 is the O.D. value of the DPPH solution without any test sample, A_2 is the O.D. value of the DPPH solution with any test sample (extracts and positive controls). The quantity or amount or

concentration of antioxidants in extracts of any living material is necessity to remove 50% of free radicals from the living system is called inhibitory concentration (IC50). All experiments were repeated five times (n=5). [24~~5~~, 36~~7~~]

Protease inhibitor assay (%)

Protease inhibitor assay was very useful to determine the anti- inflammatory activity of acetone extract of ~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf. In this assay, 2 mL of acetone extract of~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf was used at various concentrations (10 – 1000 µg/mL) added to the reaction mixture (0.05 mg trypsin, 0.5 mL of 20 mMtris-HCl buffer, pH 7.2 ± 0.2) and then allowed to stand for 10 minutes at 37 ˚C. Further incubation was carried for 15 minutes at 37 ˚C after addition of casein (1 mL, 0.8% (w/v)). By adding perchloric acid (2 mL of 70 %), the reaction was further stopped and followed by the centrifugation, the supernatant O.D. values were taken at 275 nm. The following formula determines the inhibition activity of protease.

$$\text{Protease inhibitor assay (\%)} = [(A_1\text{-}A_2)/A_1] \times 100$$

Where A_1 is the O.D. value of the blank solution (buffer) without any test sample, A_2 is the O.D. value of the test sample (extract). All experiments were repeated five times (n=5). [33~~4~~, 37~~8~~, 38~~9~~]

Assay (activity) of reducing power (%)

In assay (activity) of reducing power method, 1 mL of various concentrations (10 – 1000 µg/mL) of acetone extract of ~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf was used and added to phosphate buffer saline (PBS, 2.0 mL, 0.2 M, pH 6.3 ± 0.2) and potassium ferricyanide [$K_3Fe(CN)_6$, 2.0 mL, 1%] mixture and then allowed to stand at 475 ˚C for 30 minutes. Further, 2.0 mL of 20% trichloroacetic acid was added to the above reaction mixture followed by a centrifuge at 3500 rpm for 15 minutes. After centrifugation, the supernatant was collected and added to another reaction mixture [2 mL of D.W. and 1 mL of 0.1% ferric chloride ($FeCl_3$)]. Subsequently, the O.D. values were determined at 705 nm. Quercetin, Gallic acid and Ascorbic acid were used as standard biomolecules. All experiments were repeated five times (n=5). Finally, increase the O.D. increase the activity of reducing power (%). [33, 40,41]

Statistical data analysis

All experiments were repeated five times (n=5). SPSS V.18 software was used to analyze all statistical data in this study and all data was expressed as a mean (M) ± standard error means (SEM). The *P* value was set at *P*= 0.05. [42]

RESULTS AND DISCUSSION

Qualitative phytochemical analysis

Qualitative analysis of secondary (2^0ry) metabolites of acetone extracts of ~~*Brassica* *B.juncea* L.~~ leaf was showed in Table 1. Phenols (Ferric Chloride test), Saponins (Froth test) and Tannins (Ferric Chloride test) were present strongly (++), Alkaloids (Mayer's test), Carbohydrates (Molisch's test), Flavonoids (Alkaline reagent test) and Proteins (Xanthoproteic test) were present moderately (+) and Glycosides (Legal's test) were absent (-) in acetone extract of ~~*Brassica* *B.juncea* L.~~ leaf respectively. The symbols were used in this Table 1 represent the presence or absence of phytochemicals in acetone extracts of ~~*Brassica* *B.juncea* L.~~ leaf. ++, represents strong presence of phytochemicals; +, represents moderate (weak) presence of phytochemicals; and -, represents absence of phytochemicals respectively.

Quantitative phytochemical analysis

Total flavonoid content was expressed in micrograms of quercetin equivalents per milligram of fresh extract (µg QE/mg extract). The flavonoid content in acetone extracts of ~~*Brassica* *B.juncea* L.~~ leaf was 5.64 ± 00.09 µg/mg. Quercetin was used as a standard flavonid compound. Glucose concentration was represented in micrograms of N-acetyl glucosamine equivalents per milligram of fresh extract (µg GlcNAcE/mg extract). Glucose concentration was 97.74 ± 08.95 µg/mg in acetone extract of~~*Brassica* *B.juncea* L.~~ leaf. N-acetyl glucosamine was used as a standard sugar molecule (Table 2). Total phenol quantity in acetone extracts of ~~*Brassica* *B.juncea* L.~~ leaf was 120.15 ± 15.58 µg/mg. Total phenol content was expressed in micrograms of gallic acid equivalents per milligram of fresh extract (µg GAE/mg extract). Gallic acid used as a standard molecule. Protein content was quantified in acetone extracts of *B.*~~*rassica*~~*juncea*~~L.~~ leaf was 398.42 ± 25.15 µg/mg and expressed as micrograms of bovine serum albumin equivalents per milligram of fresh extract (µg BSAE/mg extract). Bovine serum albumin (BSA) was used as standard protein molecule. Total antioxidant activity was estimated in acetone extract of *B*~~*rassica* *juncea* L.~~ leaf was 95.26 ± 07.85 µg/mg. Total antioxidant activity was expressed in micrograms of ascorbic acid equivalents per milligram of fresh extract (µg AAE/mg extract). Ascorbic acid was used as standard molecule (Table 2). All the experiments were done five times (n=5).

Invitro studies

Antioxidant scavenging activity studies

Invitro antioxidant scavenging activity studies were performed by using NO, DPPH and ABTS activity assays respectively. All the experiments were done five times (n=5).

NO activity

NO scavenging activity of acetone extracts of *Brassica juncea* L. leaf was estimated at different concentrations (10 – 1000 µg/mL) and compared with standard molecules such as gallic acid, quercetin and ascorbic acid scavenging activities at different concentrations (10 – 1000 µg/mL). The scavenging activity was expressed in % of scavenging activity. At 10, 50, 250, 500 and 1000 µg/mL concentrations, the NO scavenging activity of acetone extracts of *Brassica juncea* L. leaf was found 10 ± 0.42%, 18.5 ± 2.85%, 28.95 ± 4.75%, 40.58 ± 7.98 and 57.84 ± 8.14% respectively whereas the standard molecules such as gallic acid (12 ± 1.02%, 27 ± 2.24%, 39 ± 3.16%, 47 ± 3.98% and 54 ± 5.74%), quercetin (14 ± 0.98%, 35 ± 2.58%, 48 ± 3.47%, 56 ± 4.85% and 61 ± 6.01%) and ascorbic acid (20 ± 1.09%, 45 ± 4.15%, 57 ± 5.08%, 67 ± 6.24% and 76 ± 7.06%) respectively (Figure 2). All concentrations (50, 250, 500 and 1000 µg/mL) of acetone extract of *Brassica juncea* L. leaf were showed significant NO scavenging activity except 10 µg/mL when compared with standard molecules (gallic acid, quercetin and acorbic acid) (Figure 2). The *P* value was < 0.05 and the $F_{(3, 19)}$ values were 2.00, 3.70, 8.22, 13.81 and 22.18 respectively.

DPPH activity

DPPH scavenging activity of acetone extracts of *Brassica juncea* L. leaf was estimated at different concentrations (10 – 1000 µg/mL) and compared with standard molecules such as gallic acid, quercetin and ascorbic acid scavenging activities at different concentrations (10 – 1000 µg/mL). The scavenging activity was expressed in % of scavenging activity. At 10, 50, 250, 500 and 1000 µg/mL concentrations, the DPPH scavenging activity of acetone extracts of *Brassica juncea* L. leaf was found 7 ± 0.32%, 22 ± 3.85%, 41 ± 5.75%, 48 ± 6.98 and 59 ± 9.14% respectively whereas the standard molecules such as gallic acid (14 ± 2.02%, 28 ± 2.64%, 45 ± 4.16%, 57 ± 5.98% and 61 ± 6.74%), quercetin (10 ± 1.48%, 27 ± 2.68%, 35 ± 3.67%, 51 ± 5.85% and 57 ± 6.51%) and ascorbic acid (15 ± 1.69%, 38 ± 5.15%, 59 ± 6.08%, 64 ± 7.24% and 73 ± 8.06%) respectively (Figure 3). All concentrations (50, 250, 500 and 1000

µg/mL) of acetone extract of *Brassica juncea* L. leaf were showed significant DPPH scavenging activity except 10 µg/mL when compared with standard molecules (gallic acid, quercetin and acorbic acid) (Figure 3). The *P* value was < 0.05 and the $F_{(3, 19)}$ values were 0.87, 1.16, 4.12, 3.23 and 5.92 respectively.

ABTS activity

ABTS scavenging activity of acetone extracts of *Brassica juncea* L. leaf was estimated at different concentrations (10 – 1000 µg/mL) and compared with standard molecules such as gallic acid, quercetin and ascorbic acid scavenging activities at different concentrations (10 – 1000 µg/mL). The scavenging activity was expressed in % of scavenging activity. At 10, 50, 250, 500 and 1000 µg/mL concentrations, the ABTS scavenging activity of acetone extracts of *Brassica juncea* L. leaf was found 9 ± 0.82%, 23 ± 3.45%, 37 ± 4.75%, 47 ± 5.98 and 51 ± 8.14% respectively whereas the standard molecules such as gallic acid (16 ± 2.42%, 34 ± 2.74%, 47 ± 5.16%, 59 ± 6.18% and 63 ± 6.64%), quercetin (10 ± 1.48%, 27 ± 2.68%, 38 ± 3.67%, 51 ± 5.85% and 57 ± 6.51%) and ascorbic acid (19 ± 1.89%, 43 ± 5.75%, 59 ± 6.28%, 67 ± 7.34% and 76 ± 8.66%) respectively (Figure 4). All concentrations (50, 250, 500 and 1000 µg/mL) of acetone extract of *Brassica B.juncea* L. leaf were showed significant ABTS scavenging activity except 10 µg/mL when compared with standard molecules (gallic acid, quercetin and acorbic acid) (Figure 4). The *P* value was < 0.05 and the $F_{(3, 19)}$ values were 1.94, 2.00, 4.08, 5.15 and 7.48 respectively.

IC50 values

IC50 values of acetone extract of *Brassica B.juncea* L. leaf was compared with standard molecules (gallic acid, quercetin and ascorbic acid). NO (785.25 ± 15.85), DPPH (615.29 ± 12.54) and ABTS (975.85 ± 23.12) IC50 values of extract were showed significant (*P*< 0.05) variation when compared with standards (Table 4). EC50 values determines the scavenging activity strength of the compound, the lowest EC50 values showed greater strength in scavenging activity of free radicals. IC50 values were expressed in µg/mL. All the experiments were done five times (n=5).

Protease inhibitor assay (%)

Protease inhibitor activity (%) of extract was showed in Figure 5. The protease inhibitor activity of extract at different concentrations/doses were 4.64 ± 1.09 (10 µg/mL), 8.57 ± 2.38 (50 µg/mL), 25.87 ± 5.38 (250

µg/mL), 39.75 ± 5.89 (500 µg/mL) and 41.28 ± 7.28 (1000 µg/mL) respectively. Dose/Concentration wise the protease inhibitor activity was increased significantly (Figure 5). The results showed that acetone extract of ~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf has a greater protease inhibitor activity as well as invitro anti-inflammatory activity.

Assay (activity) of reducing power (%)

The reducing power (%) was increased significantly as the concentration of extract was increased. The reducing power (%) of acetone extracts of ~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf was 50.26 ± 05.68 at 10 µg/mL, 138.95 ± 10.58 at 50 µg/mL, 280.75 ± 15.67 at 250 µg/mL, 387.95 ± 21.54 at 500 µg/mL and 537.95 ± 26.58 at 1000 µg/mL respectively whereas the reducing power (%) of standard molecules such as gallic acid (65.87 ± 06.02 at 10 µg/mL, 154.63 ± 09.54 at 50 µg/mL, 357.85 ± 12.65 at 250 µg/mL, 734.12 ± 23.54 at 500 µg/mL and 859.21 ± 32.54 at 1000 µg/mL), quercetin (79.35 ± 06.58 at 10 µg/mL, 168.65 ± 16.58 at 50 µg/mL, 368.25 ± 21.24 at 250 µg/mL, 756.24 ± 31.25 at 500 µg/mL and 865.31 ± 34.65 at 1000 µg/mL) and ascorbic acid (55.85 ± 05.84 at 10 µg/mL, 156.25 ± 09.54 at 50 µg/mL, 310.24 ± 15.42 at 250 µg/mL, 412.57 ± 21.30 at 500 µg/mL and 546.32 ± 25.84 at 1000 µg/mL) respectively (Table 3). Based on results, acetone extract of ~~*Brassica* *B.*~~ *juncea*~~L.~~ leaf was showed greater reducing activity. All the experiments were done five times (n=5).

The uses of medicinal plants are practiced since 3000 years ago, [21, 43] ~~*Brassica* *B.*~~ *juncea*~~L.~~ used as diet as well as medicinal plant throughout the world especially in Asian countries such as China, Japan, India and European countries.The current work focused on qualitative and quantitative phytochemical constituents, antioxidant and anti inflammatory activities of acetone extract of *B*~~*rassica*~~ *juncea*~~L.~~ leaf.

Presence of qualitative (alkaloids, carbohydrates, flavonoids, phenols, proteins, saponins and tannins) and quantitative [(flavonoid content (Quercetin), glucose concentration (N-acetyl glucosamine), phenolic content (Gallic acid), protein content (Bovine serum albumin) and Total antioxidant activity (Ascorbic acid)] phytochemical constituents in acetone extracts of *B*~~*rassica*~~ *juncea*~~L.~~ leaf are reasonable for its medicinal uses against asthma, blood pressure, restore normal sleep pattern, atherosclerosis, diabeties,anticonvulsant activity and heart diseases. [12-15, 44]

Based on chemical nature, there are various types of total flavonoids (flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones) are present in environment. Nowadays these are gaining most importance, to treat number of diseases (antiviral, anti-allergic, antiplatelet, antiinflammatory, antitumor and antioxidant activities). Flavonoids (Quercetin) have been quantified in acetone extract of *B. rassica juncea* L. leaf which in turn increases reduced glutathione levels (GSH) and decreases malondialdehyde (MDA) levels in body fluids.[45-47] In Brassicaceae family vegetables, glucose content is more and released by the action myrosinase enzyme on sinigrin. Scavenging activity of plant phenolic acids of acetone extract of *B. rassica juncea* L. leaf may be the basis to the human health benefits. [27,28] Thus, quantification and later identification of phenolic acids can give important information coupled to the antioxidant functions and likely health benefits of *B. rassica juncea* species. The presence of secondary metabolites in acetone extracts of *B. rassica juncea* L. leaf are bioactive compounds and may responsible for antioxidant properties. Hence, it could be strongly recommended as a sustainabledrug due to chelating properties of bioactive compounds. [15,28, 34, 48-50]

To determine the antioxidant properties of the acetone extracts of *B. rassica juncea* L. leaf., NO, DPPH, ABTS, Protease inhibitor assay (%) and reducing power in vitro studies were used via colour intensity measurements. Change in colour is proportional to the antioxidants concentration. A low O.D values of reaction mixures great free radical scavenging properties. [51] Antioxidant activitieswere observed in the acetone extracts of *B. rassica juncea* L. leaf as dose-dependent manner. This study results suggest that the acetone extracts of *B. rassica juncea* L. leaf contain secondary metabolites which can scavenge free radicals and avert possible damage that can linked with the phenolic acids present in extracts. Hence, phyto compounds have the property to counteract the effect of NO formation and nitrosative stress, which is produced endothelial cells, macrophages, neurons, etc. which in turn involved in various physiological processes regulation. According to Han SS, 2004 *B. rassica juncea* has DPPH scavanging activity is 42µg/ml. [52] This does not deviate so far from our study.

Enzymes and proteins play a major role in inflammation and different functions of the immune system. Proteolytic enzymes (bromelain, papain, trypsin and chymotrypsin) are necessary regulators and modulators of the inflammatory response. [53] Trypsin enzyme has been shown to induce in

vivo epidermic proliferation, vasodilatation and inflammatory infiltration within the higher epidermis by the activation of PAR2 family. The expression of PAR2 on epithelium cells and inflammatory cells together with neutrophils and macrophages, determines the involvement of PAR2 in each pro-inflammatory and anti inflammatory responses of various experimental models of inflammation. [54] An earlier report indicates flavonoid to be a competitive substance of trypsin. [55]Since *B. rassica juncea*contains flavonoids that are liable for inhibition of trypsin enzyme and therefore act as anti-inflammatory agent.

The reducing power enhanced because the extract concentration enhanced, indicating some compounds within the extracts is electron donor and will react with free radicals to convert them in to stable merchandise and to terminate radical chain reactions. According to Shimada et al., 1992, the antioxidant activity has been reported to be concomitant with the event of reducing power. The reducing power of the ethanolic extracts of the plant may be because of its hydrogen donating ability. [56]

It was already observed and established that phenolics and flavonoids of *B. juncea* have great health benefits, therapeutic potentials and antioxidant properties and good source of phenolic compounds than that of any other plant in the Brassicaceae family. [57]While outcomes of this study confirmed that dose dependent antioxidant activity, it is clear that the acetone extracts of *Brassica B. juncea*L. leaf displayed low radical scavenging capabilities contrasted with the control (quercetin, gallic acid and ascorbic acid).

CONCLUSION

Finally, active compounds of acetone extracts of *B. rassica juncea*L. leaf may be credited to the phytochemicals that they contain. The phenolic and flavonoid contents in the acetone extracts of *B. rassica juncea*L. leaf signify that a potential source of antioxidants. The acetone extracts of *Brassica juncea*L. leaf showed potential antioxidant properties and are able of scavenging ROS. The in vitro antioxidant properties of the acetone extracts of *Brassica B. juncea*L. leaf signify that they could be used to prevent oxidative stress and their related diseases. But, a more explorations are required to know the phytocompounds and their antioxidant properties.

CONFLICTS OF INTEREST

All the authors were declared that thers is no conflict of interest.

Table 1. Qualitative investigation of phytochemical constituents in acetone extracts of *Brassica juncea* L. leaf.

Serial number	Name of the compound	Test name	Inference
1.	Alkaloids	Mayer's test	+
2.	Carbohydrates	Molisch's test	+
3.	Flavonoids	Alkaline reagent test	+
4.	Glycosides	Legal's test	-
5.	Phenols	Ferric Chloride test	++
6.	Proteins	Xanthoproteic test	+
7.	Saponins	Froth test	++
8.	Tannins	Ferric Chloride test	++

Notes: Negative symbol (-) represents: a specific phytochemicals compound was absent in acetone extracts of ~~*Brassica*~~*B. juncea* ~~L.~~ leaf; Single positive symbol (+) indicates: a particular phytocomponents was present moderately (weakly) in acetone extracts of ~~*Brassica*~~*B. juncea* ~~L.~~ leaf; Double positive symbol (++) denotes: strong presentation of a particular phytoconstituents in acetone extracts of ~~*Brassica*~~*B. juncea* ~~L.~~ leaf with respect to specific tests.

Table 2. Quantitative analysis of phytochemical constituents (biomolecules) in acetone extracts of ~~*Brassica*~~*B. juncea* ~~L.~~ leaf.

Serial number	Biomolecules with their respective standard molecules	Quantity (amount) present in acetone

		extracts of ~~*Brassica*~~ *B.juncea*~~L.~~ leaf. (µg/mg extract)
1.	Flavonoid content (Quercetin)	5.64 ± 00.09
2.	Glucoseconcentration (N-acetyl glucosamine)	97.74 ± 08.95
3.	Phenolic content (Gallicacid)	120.15 ± 15.58
4.	Protein content (Bovine serum albumin)	398.42 ± 25.15
5.	Total antioxidant activity (Ascorbic acid)	95.26 ± 07.85

Notes: Statistical data is expressed as mean (M) ±standard error mean (SEM). Where n=5. The content of biomolecules inacetone extractsof ~~*Brassica*~~*B.juncea*~~L.~~ leaf. Was expressed in microgram (µg) per milligram (mg).

Table 3. Estimation and comparison of reducing power (%) of test sample (*B. ~~rassica~~juncea~~L.~~* acetone extract) and standard samples (Gallic acid, Quercetin and Ascorbic acid) at various concentration (doses) levels.

Reducing power (%) at different concentrations (µg/mL)	**Reducing power (%)**			
	Samples			
	~~Brassica~~ B.juncea~~L.~~ **acetone extract (Test sample)**	**Gallic acid (Standard)**	**Quercetin (Standard)**	**Ascorbic acid (Standard)**
10µg/mL	50.26 ± 05.68	65.87 ± 06.02	79.35 ± 06.58	55.85 ± 05.84
50µg/mL	138.95 ± 10.58	154.63 ± 09.54	168.65 ± 16.58	156.25 ± 09.54
250µg/mL	280.75 ± 15.67	357.85 ± 12.65	368.25 ± 21.24	310.24 ± 15.42
500µg/mL	387.95 ± 21.54	734.12 ± 23.54	756.24 ± 31.25	412.57 ± 21.30
1000µg/mL	537.95 ± 26.58	859.21 ± 32.54	865.31 ± 34.65	546.32 ± 25.84

Notes: Statistical data is expressed as mean (M) ±standard error mean (SEM). Where n=5.

Table 4. Estimation and comparison of IC50 and EC50 values of the acetone extracts of *Brassica B. juncea* L. leaf and with standards (Gallic acid, Quercetin and Ascorbic acid).

Samples	NO		DPPH		ABTS	
	IC50 (µg/mL)	1/EC50 (µg/mL)	IC50 (µg/mL)	1/EC50 (µg/mL)	IC50 (µg/mL)	1/EC50 (µg/mL)
Brassica B. juncea L.	785.25 ± 15.85	0.001 ± 0.001	615.29 ± 12.54	0.0016 ± 0.001	975.85 ± 23.12	0.001 ± 0.001
Gallic acid	575.08 ± 10.75	0.001 ± 0.001	347.85 ± 07.94	0.0028 ± 0.001	305.89 ± 09.87	0.003 ± 0.001
Quercetin	270.18 ± 05.87	0.003 ± 0.001	485.54 ± 08.56	0.0020 ± 0.001	485.14 ± 11.24	0.002 ± 0.001
Ascorbic acid	60.54 ± 03.19	0.017 ± 0.030	53.47 ± 03.24	0.0180 ± 0.006	55.15 ± 03.25	0.018 ± 0.004

Notes: NO: Nitric oxide; DPPH: 1, 1-diphenyl-2-picrylhydrazyl; ABTS: 2, 2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid; IC50: Inhibitory concentration 50%; EC50: Effective concentration 50% . All statistical data is showed in mean (M) ± standard error mean (SEM). Where n=5.

Fig. 1. *Brassica B. juncea* L. herb. Arrow symbol shows the young and tender leaves of *Brassica B. juncea* L. herb.

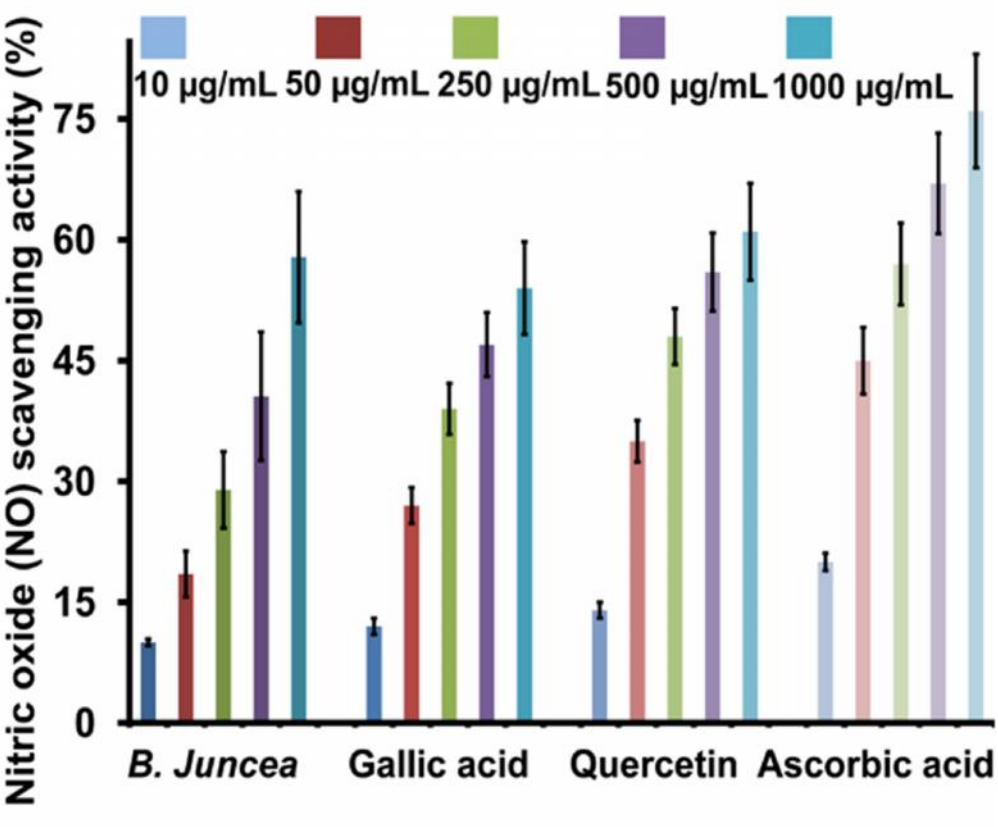

Fig. 2. Nitric oxide (NO) scavenging activities (%) of acetone crude extractof ~~*Brassica*~~ *B.juncea*L. leaf with different concentrations (10µg/mL, 50µg/mL, 250µg/mL, 500µg/mL and 1000µg/mL). Data are represented as mean (M) ± standard error mean (SEM) (n = 5). Gallic acid, Quercetin and Ascorbic acid were used as a standard biomolecules at a concentration levels (10µg/mL, 50µg/mL, 250µg/mL, 500µg/mL and 1000µg/mL). Different colour bars showed different concentrations of acetone crude extractof ~~*Brassica*~~ *B.juncea*L. leaf, Gallic acid, Quercetin and Ascorbic acid respectively.

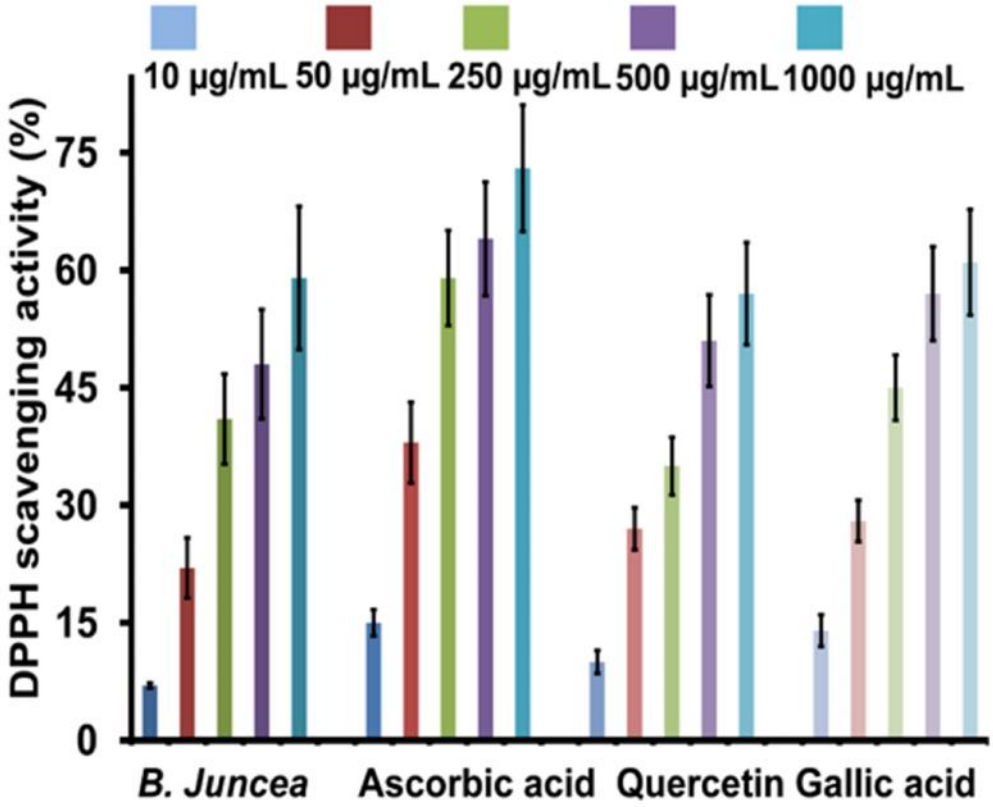

Fig.3.1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activities (%) of acetone crude extractof ~~*Brassica* B~~ *juncea*L. leafwith different concentrations (10µg/mL, 50µg/mL, 250µg/mL, 500µg/mL and 1000µg/mL). Data are represented as mean (M) ± standard error mean (SEM) (n = 5). Gallic acid, Quercetin and Ascorbic acid were used as a standard biomoleculesat a concentration levels (10µg/mL, 50µg/mL, 250µg/mL, 500µg/mL and 1000µg/mL).Different colour bars showed different concentrations of acetone crude extractof ~~*Brassica* B~~ *juncea*~~L.~~leaf, Gallic acid, Quercetin and Ascorbic acid respectively.

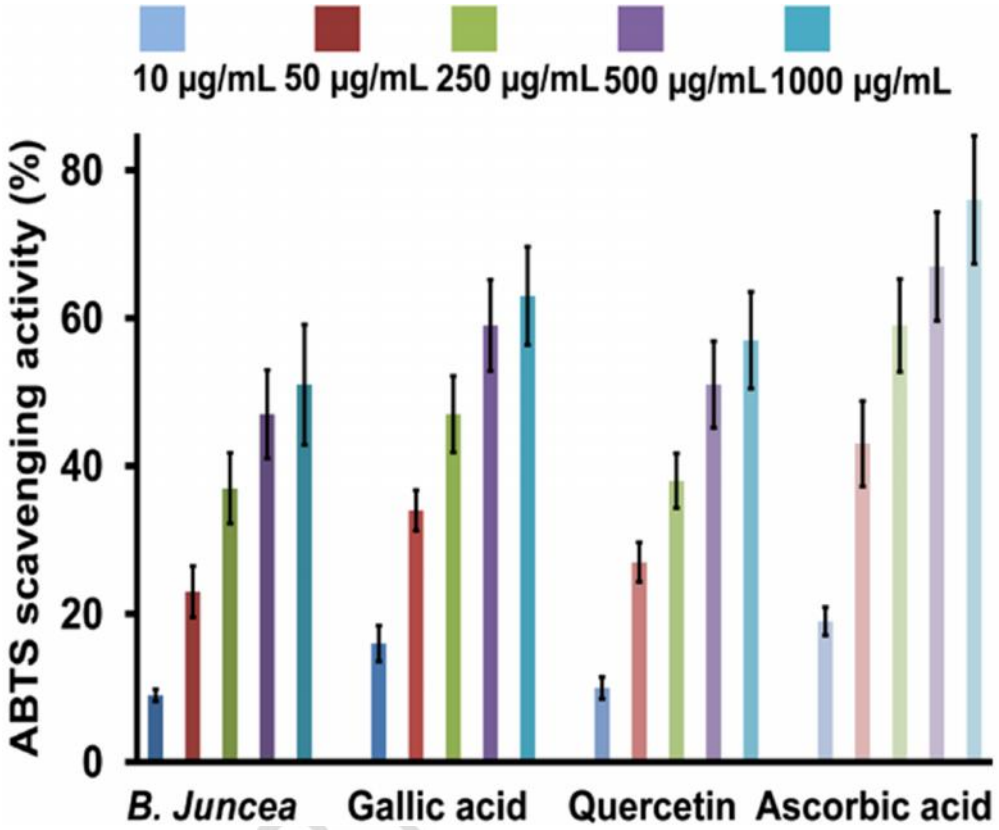

Fig.4.2, 2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) scavenging activities (%) of acetone crude extractof ~~*Brassica* *B.*~~ *juncea*~~L.~~leafwith different concentrations (10µg/mL, 50µg/mL, 250µg/mL, 500µg/mL and 1000µg/mL). Data are represented as mean (M) ± standard error mean (SEM) (n = 5). Gallic acid, Quercetin and Ascorbic acid were used as a standard biomoleculesat a concentration levels (10µg/mL, 50µg/mL, 250µg/mL, 500µg/mL and 1000µg/mL).Different colour bars showed different concentrations of acetone crude extractof ~~*Brassica* *B.*~~ *juncea*~~L.~~leaf, Gallic acid, Quercetin and Ascorbic acid respectively.

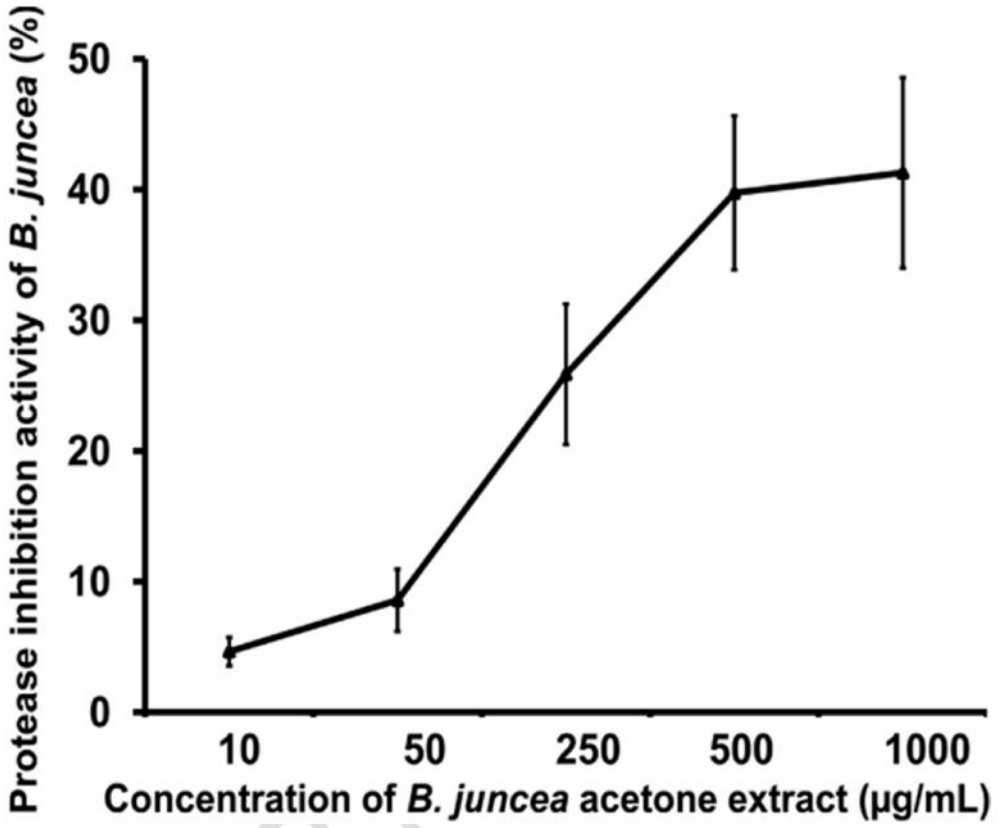

Fig.5. Protease inhibition activities (%) of acetone extract of *B. juncea*~~*(Brassica juncea*L.)~~ leaf with concentrate (dose) dependent manner (10µg/mL, 50µg/mL,250µg/mL,500µg/mL and 1000µg/mL). Statistical analysis data are expressed as mean (M) ± standard error mean (SEM) (n = 5).

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