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Research Article

Phytochemical Profiling and Characterization of Bioactive Compounds from *Brassica oleracea.*

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ABSTRACT

Phytochemicals are non-nutrient plant chemical compounds or bioactive components and are responsible for protecting the plant against microbial infections. *Brassica* family as secondary metabolite contains tannins, phenols, steroids, terpenoids, flavonoids and glucosinolates. Glucosinolates(GLS) are bioactive compounds in crucifers associated with cancer protection. Glucosinolates are known to possess fungicidal, bacteriocidal, nematocidal and allelopathic properties. *Brassica* family contains highest quantity of phenolic compounds that exhibit the greatest anti-oxidant activity. Antioxidants play a key role in preventing generation of free radicals. The present study was designed to screen plant secondary metabolites from broccoli along with determination of glucosinolates, antioxidant activity and antibacterial activity assays.

Keywords: *Brassica oleracea*, Glucosinolates, Antioxidants

INTRODUCTION

Plants are a resource for a variety of drugs such as emetics, anti-cancer, antimicrobials etc. Phytochemicals play a vital role as they exhibit these propertie[1]. Broccoli (*Brassica oleracea var. italica)* belongs to the Cruciferaceae family. It is high in complex carbohydrates and rich in antioxidants. It also contain beta-carotene, lutein and carotenoids. It is also a good source of vitamin C, vitamin K and an excellent source of folate[2].

A high intake of cruciferous vegetables is associated with a reduced risk of cancer, particularly lung and those of the gastrointestinal tract[3]. Broccoli contains many bioactive components including flavonoids, minerals and vitamins. The most-studied bioactive compounds in crucifers associated with cancer protection are glucosinolates(GLS). Glucosinolates are β-D-thioglucoside-N-hydroxysulfates. More than 120 individual glucosinolates have been identified. The diversity is found in the R Group which leads to a wide variation in the polarity and biological activity of the natural products. Glucosinolates generally occur in the form of sodium or potassium salt[4]. Sinigrin is a glucosinolate found in cruciferous vegetables and hydrolyzed by myrosinase upon injury or mechanical disruption of the plant tissue.The hydrolysis products include allyl isothiocynate, allyl thiocynate, allyl cyanide. Each compound contributes to flavour and characteristic aroma in such plants[5].

These secondary metabolites induce detoxification of carcinogens, limit production of cancer related hormones, block carcinogens and prevent tumor growth and therefore it is suggested to consume a diet rich in fruits and vegetables, especially cruciferous vegetables such as broccoli, so as to reduce the risk of developing several types of cancers[6].

MATERIALS AND METHODS

Collection of Broccoli

Broccoli was purchased from a local grocer. The leaves were washed and air dried in shade and ground to a fine powder using an electric grinder.

Extraction

The powder obtained was used for extraction. Extraction was performed by following two methods

Soxhlet Extraction

10g powder of Broccoli was extracted in hexane, methanol, acetone and water (with increasing solvent polarity)[7]. After extraction, the solvent was evaporated and extract was preserved at 4˚C. For phytochemical screening, extracts were dissolved in distilled water.

Cold Maceration

10 gm of powdered Broccoli in methanol (250ml) was kept for 3 days on a rotary shaker. The supernatant obtained was utilized for phytochemical screening[7].

Phytochemical screening

Qualitative determination of phytochemicals

Tests for Tannins

In a small quantity of test residue, distilled water was added and the solution was warmed and filtered. This filtrate was used for the tannin content determination.

Ferric chloride test[8] and lead acetate test[9] for tannins were performed according to standard procedures.

Tests for Flavanoids

A small quantity of test residue is dissolved in 5 ml

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Table 1: Percent Yield from Soxhlet extracted and Cold macerated samples of broccoli

Plant	Treatment	Solvent	Weight in grams	% Yield
Broccoli	Soxhlet	1.Hexane	1.3	13%
		2.Acetone	0.5	5%
		3.Methanol	2.5	25%
		4.Water	1.5	15%
	Cold maceration	1.Methanol	3	30%

Table 2: Concentration of total phenol as Gallic acid equivalent per gram of plant material

Sample	Broccoli (Soxhlet)	Broccoli (Cold macerated)
Concentration of Phenol in µg/ml	155.5	143.3

Table 3: Dry weight of Alkaloids in grams

Sample	Broccoli (Soxhlet)	Broccoli (Cold macerated)
Dry weight of Alkaloids in grams	0.504	0.604

Table 4: Flavonoid concentration as Quercetin equivalent per gram of plant material

Sample	Broccoli (Soxhlet)	Broccoli (Cold macerated)
Concentration of Flavonoid in µg/ml	750	680

Table 5: Dry weight of Saponins in grams

Sample	Broccoli (Soxhlet)	Broccoli (Cold macerated)
Dry weight of Saponins in grams	0.650	0.400

Table 6: Anthocyanidinconcentration as Catechin equivalent per gram of plant material

Sample	Broccoli (Soxhlet)	Broccoli (Cold macerated)
Concentration of Anthocyanidin in µg/ml	140	200

Table 7: Concentration of Tannin per gram of plant material

Sample	Broccoli (Soxhlet)	Broccoli (Cold macerated)
Concentration of Tannin in µg/ml	0.64	1.84

ethanol (95%) and treated with few drops of concentrated HCl and 0.5 g Mg. A pink or magenta color develops within 3 minutes. This is indicative of presence of flavonoids[8].

Tests for Phenols

Test for phenol included Ferric chloride test[8] and Nitric acid test[9]. These were also performed by standard procedures.

Tests for Carbohydrate

Molisch's test[10] was performed for carbohydrate determination in broccoli.

Test for Anthaquinone

Test for anthaquinone included Borntrager's test[8]

Test for steroids

Salkowski's test was performed for testing steroids[8].

Quantitative determination of phytochemicals

Determination of phenols

1000μg extract + 1mL Folin-Ciocalteu reagent + shake + wait for 3 min + 3mL 2% Na_2CO_3 + keep for 2 hr. Read absorbance at 760 nm. Gallic acid was utilized as standard[8].

Determination of alkaloids

5g sample + 200 ml 10% acetic acid in ethanol + cover it and incubate for 4hr + filter. Extract + keep on boiling water bath to remain one fourth original volume + drop wise ammonium hydroxide until complete precipitation + allow the solution to settle + collect precipitate + wash with dilute NH_4OH + filter. Take residue, dry it and weigh[8].

Determination of flavonoids

10g sample extracted with 100mL 80% aqueous methanol + filter through whatman filter paper no. 42 (125mm). Transfer filtrate into crucible + evaporate + weigh. 0.5 ml each plant extracts + 1.5 ml of methanol + 0.1 ml of 10% aluminum chloride + 0.1 ml of 1 M potassium acetate + 2.8 ml of distilled water + keep at room temperature for 30 minutes. Read at 415 nm with UV/Visible spectrophotometer. Total flavonoids contents were calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg/ ml in methanol[8].

Determination of anthocyanidin

1mL of sample or catechin standard solutions (50-300 mg/L) + 2.5mL of 1% (w/v) vanillin in methanol + 2.5mL of 9.0 N HCl in methanol + incubate at 30°C for 20 min + take absorbance at 500 nm[8].

Determination of tannin

50µL extract + make up to 7.5mL with water + 0.5mL Folin-Denis reagent + 1mL Na_2CO_3 + make up to 10mL with water. Read at 700nm[8].

Estimation of Sinigrin

The Sinigrin Potassium Salt procured from SRL was assayed Spectrophotometrically. The quantity of Sinigrin present in Broccoli was assayed by measuring the rate of decrease in absorbance at 227 nm. The 150µl reaction mixture contained 33mM of potassium phosphate buffer (pH 6.0), 37.5 mM Sinigrin,1.07mM EDTA[11].

Thin Layer Chromatography

TLC was performed to separate the glucosinolates present in broccoli. For preparation of slides a slurry was made which consisted of silica and plaster of paris in 4:1 ratio[13]. The slurry was then poured on the glass plate as a thin layer. The plates were air dried and kept for activation in a hot air oven at 120˚C.This served as a stationary phase. Two mobile phases were utilized: Butenol: acetic acid :

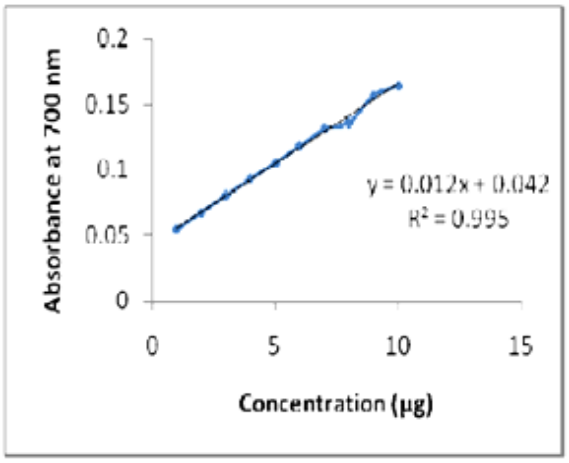


Figure 1: Gallic acid standard for Phenol

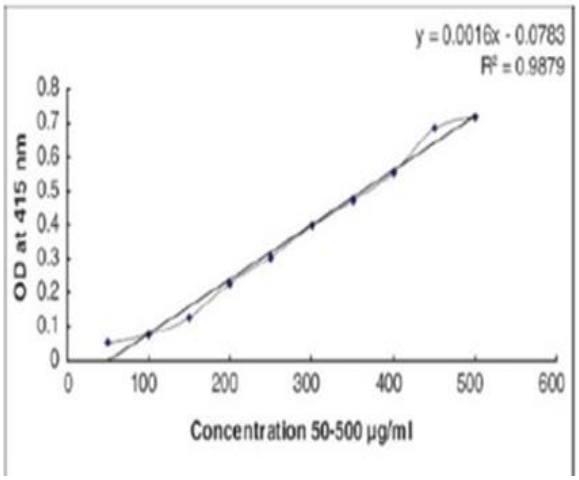


Figure 2 : Quercetin standard for flavonoids

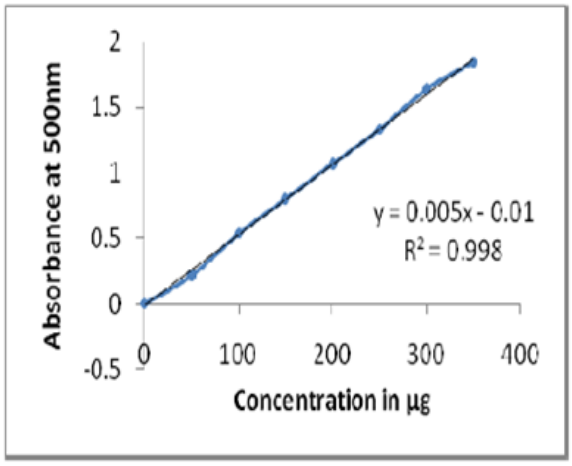


Figure 3 :Catechin standard for Anthocyanidin

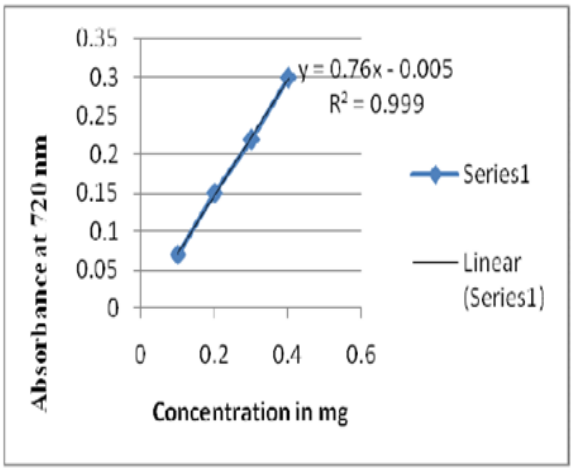


Figure 4 : Tannic acid standard for tannins

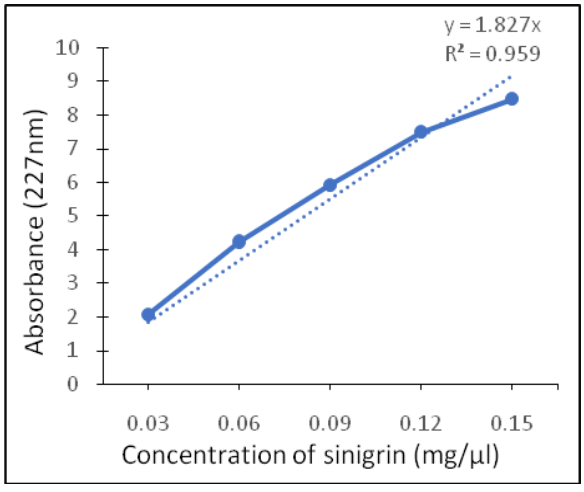


Figure 5 : Standard Sinigrin Curve

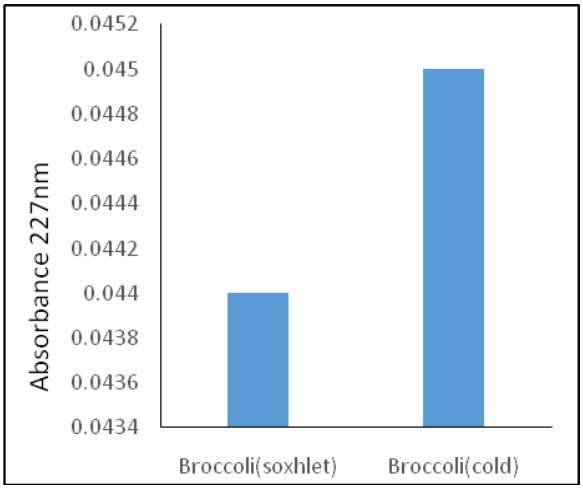
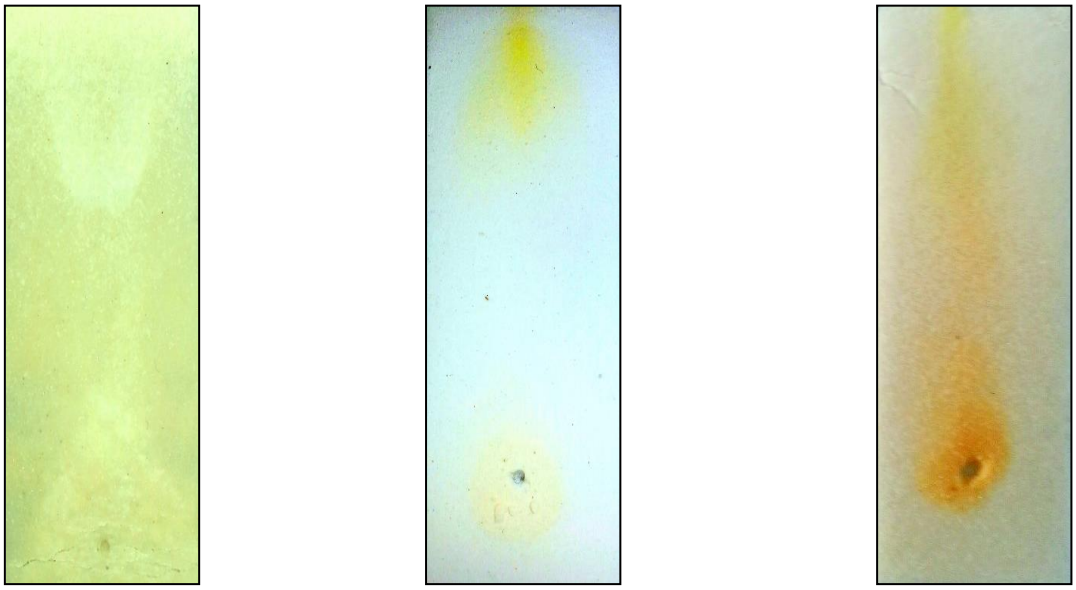


Figure 6 :Graphical comparison of broccoli extracts

water (4:1:5)[12] and Butenol : pyridine : water (6:4:2)[13]. The sample (80µl) was applied on the plates. As the sample dried was kept in a closed chamber, previously saturated with the solvent (both the solvents which are mentioned above). The process was allowed to run and dried. The plates were sprayed with ammonical silver nitrate (1 gm $AgNO_3$ + ammonia 2.5 ml which was



Standard Sinigrin | Broccoli (Cold maceration) | Broccoli (Soxhlet)

Figure 6 : Thin Layer Chromatography of Standard Sinigrin and broccoli extracts

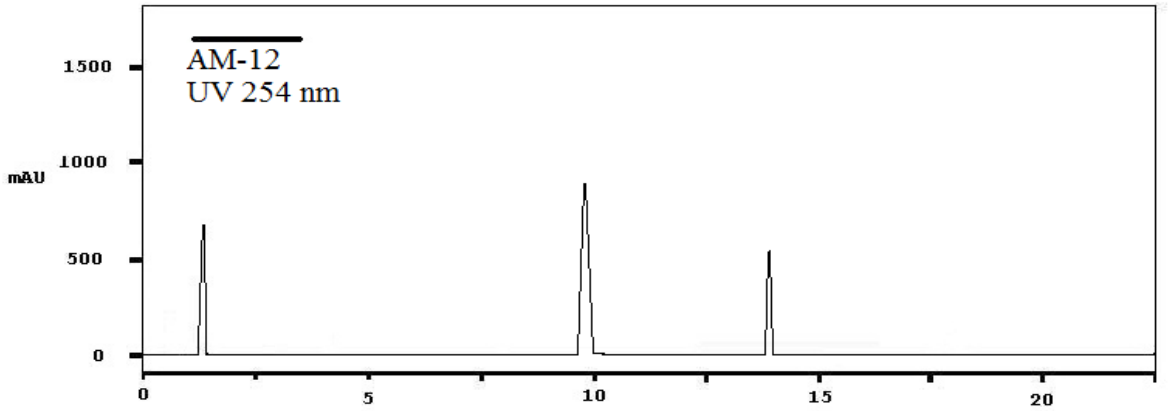


Figure 7 : HPLC for Broccoli

Table 8 : Rf values of Broccoli extracts

Extract	Phyochemical	Rf value
Broccoli (Soxhlet extract)	Glucobrassicin	0.64
Broccoli (Cold maceration)	GlucobrassicinSulphonate	0.67

Table 9 : Rt for Broccoli

Peak Number	Retention Time in Min	Glucosinolate
1	1.25	-
2	9.55	4-hydroxyglucobrassicin
3	13.45	glucosibarin

diluted to 1 litre)[14] and then were observed for spots and Rf values were calculated.

Rf= Distance travelled by solute from the base line / Distance travelled by solvent front

High performance liquid chromatography

HPLC of Broccoli extract was performed for detection and identification of bioactive compounds i.e. glucosinolates. The HPLC method provides a simple means for determination of desulphoglucosinolates. These were separated using a AminoPak C18 reverse phase column with a flow rate of 1.4 ml/min. Elution of components from the HPLC column was performed by gradient system of methanol/water (90:10, v/v,) and was detected by the UV detector at the wavelength of 254 nm[15].

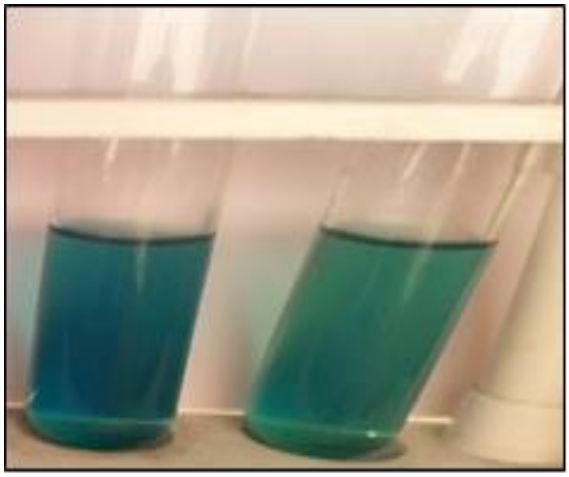


Figure 8: 1-Broccoli (Soxhlet extract),
2-Broccoli (Cold maceration).

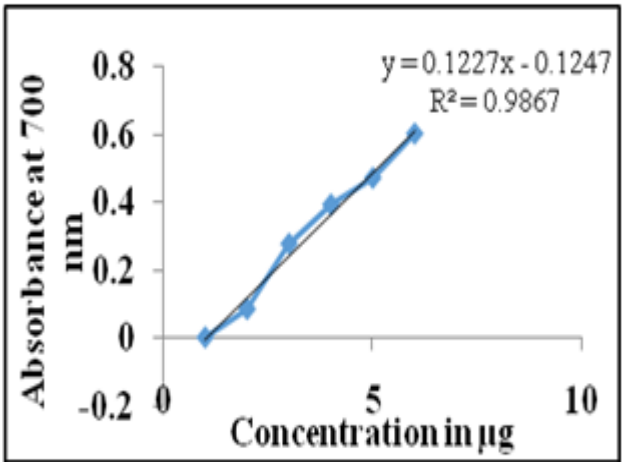
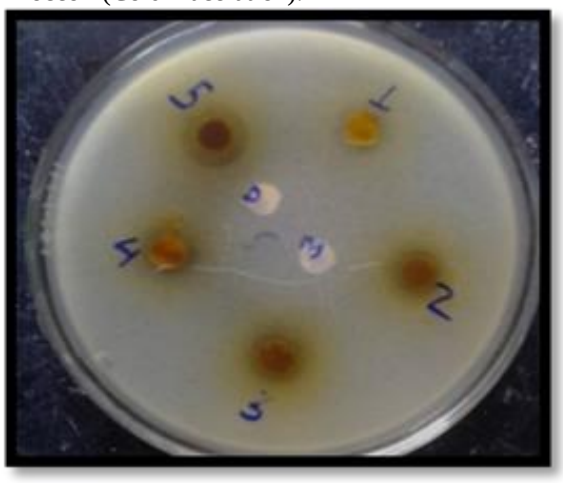
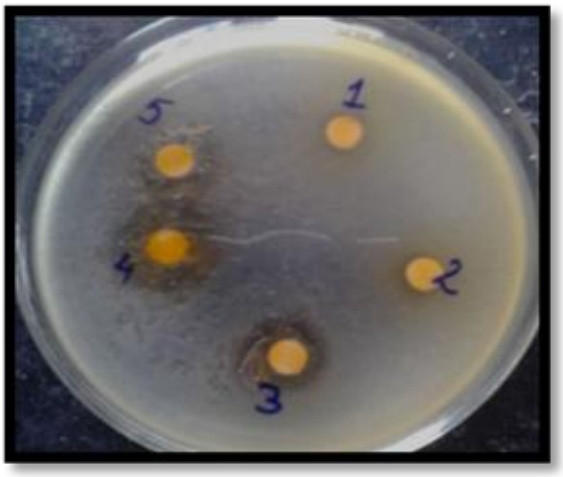


Figure 9: Ascorbic acid standard for antioxidants



Broccoli (Cold maceration)



Broccoli (Soxhlet)

Figure 10 : Antibacterial activity of broccoli extracts

Ferric Reducing Ability of Plasma (FRAP) Assay / Reducing Power Assay

Reducing power was determined by taking the sample in 1ml of methanol and then mixed with phosphate buffer (5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5 ml, 1%), and the mixture was incubated at 50°C for 20 min. Next, 5ml of trichloroaceticacid (10%) was added to the reaction mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (5 ml) was mixed with distilled water (5ml) and ferricchloride (1 ml, 1%), and the absorbance was measured at 700 nm.The reducing property of the extract was determined by taking 1ml of different dilutions of standard solutions of Ascorbic acid (10 -100 µg/ml)[16].

Antibacterial Activity

Circular discs of 5mm diameter were cut from the Whatmann filter paper no. 1. Discs were impregnated with different volume (10µl-50µl) of broccoli extract at five different concentrations (20mg/ml, 40mg/ml, 60mg/ml, 80mg/ml & 100mg/ml). The discs were aseptically placed over plates of Muller Hinton agar seeded with test pathogen (*E.coli*). 2% methanol and 0.2 % DMSO here used as control. The plates were incubated in an upright position at 37 ˚C for 24 hours[17].

RESULTS

Qualitative estimation

Qualitative estimation of phytochemicals revealed that the methanolic extract of broccoli contains high quantities of alkaloids, tannins, flavonoids, phenols and proteins whereas saponins were absent. Hexane extract of broccoli has high amount of alkaloids, phenols and proteins. Acetone and water extracts of broccoli possess little amount of sterols, alkaloids and tannins but are rich in phenols and proteins.

Cold macerated methanolic extract of broccoli contains high amount of sterols, alkaloids, tannins, phenols and proteins.

Quantitative estimation

Estimation of Sinigrin

Different concentration of standard Sinigrin was taken (i.e 1mg, 2mg, 3mg, 4mg, 5mg). The concentration of Sinigrin in broccoli was found to be 1.46mg/100g in

soxhlet extract and 1.5 mg/100g in cold macerated extract.

Thin Layer Chromatography

Thin layer chromatography was performed to separate the glucosinolates (glucoraphanin, glucobrassicin, glucoiberin,sinigrin, gluconapin and neo-glucobrassicin) and its hydrolysis products (isothiocyanates, nitriles) from broccoli extract. The Rf values were calculated using the formula;

Rf = (distance migrated by spot) / (distance migrated by solvent)

Ferric Reducing Ability of Plasma (FRAP) Assay / Reducing Power Assay

The antioxidant can donate an electron to free radicals, which leads to the neutralization of the radical. The product was visualized by forming the intense Prussian blue colour complex and then measured at λ700nm. A higher absorbance value indicates a stronger reducing power of the samples. The antioxidant activity of broccoli in soxhlet extract was found to be 3.422 ascorbic acid equivalent/gm of extract and 1.303 ascorbic acid equivalent/gm of extract in cold macerated extract.

Antibacterial Activity

The antibacterial activity was investigated for soxhlet extracts and cold macerated extracts of both cabbage and broccoli by agar disc diffusion method.

Antibacterial activity was observed in broccoli soxhlet and cold macerated extract at concentration 60mg/ml, 80mg/ml and 100mg/ml.

DISSCUSSION

Phytochemical profiling of *Brassica oleraceavaritalica*extract was performed. The resultsindicated presence of tannin, phenol, alkaloid, flavoniod and anthocyanidin. It is reported that different phytochemicals possess a wide range of activities, which may help in protection against chronic diseases. For example, alkaloids protect against chronic diseases[18]. Flavonoids are known to have activity against pathogens and therefore aid the antimicrobial activities of medicinal plants[19]. Tannin is reported to have antimicrobial activity and antibacterial activity[8].

The glucosinolate Sinigrin present in broccoli extract was measured spectrophotometrically. The absorbance was read at 227nm. The broccoli cold macerated extract contained highest amount of sinigrin that is 1.5mg/100g. The reported value is 3.23mg/100g[22]. The soxhlet extracted sample contained less amount of sinigrin as compared to cold macerated extract. The reason might be heating treatment. During heating, GSL levels are reduced by enzymatic breakdown, thermal breakdown, and leak into the hot medium. Thermal breakdown of synthetic glucobrassicin results in 10% degradation was found to be 1.5 mg/100g in cold macerated extract.

TLC and HPLC of Broccoli extract were performed for detection and identification of bioactive compounds i.e. glucosinolates. The Rf values obtained were calculated which indicated presence of different glucosinlates. We have found presence of glucobrassicin (Rf = 0.64) and glucobrassicinsulphonate (Rf = 0.67) which corresponds to standard Rf values of both glucosinolates detected[20]. HPLC analysis proved presence of 4-hydroxyglucobrassicin[21] and glucosibarin[22].

The antioxidant activity of broccoli in soxhlet extract was found to be 3.422 ascorbic acid equivalent/gm which is less than 8.303 ascorbic acid equivalent/gm of cold macerated broccoli extract. Cold macerated extract showed more potent antioxidant activity than soxhlet extract.

Antibacterial activity was observed at concentration 60mg/ml, 80mg/ml and 100mg/ml for both soxhlet and cold macerated extracts. The highest zone of inhibition in soxhlet extract was 12mm and for cold macerated extract was found to be 14mm.

Thus it can be said that the presence of antioxidant and antibacterial activity is due to presence of phytochemicals present in broccoli.

From the above results it can be concluded that the cold macerated methanolic sample possess highest amount of antibacterial and antioxidant activity which can be used for the development of novel drugs after due ADME testing.

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