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BRASSICA OLERACEA: PHYTOCHEMICAL PROFILING IN SEARCH FOR ANTICANCER COMPOUNDS

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ABSTRACT

Phytochemicals are non nutritive, naturally occurring, plant derived chemical compounds having medicinal properties. Genus Brassica contains various phytochemicals like tannins, phlobatannins, terpenoids, flavonoids, glycosides and steroids. The principle component being glucosinolate. Glucosinolates are known to possess antibacterial, antifungal as well as anticancerous activity. In this study, cabbage extract has been investigated for phytochemical screening with special focus on glucosinolates. Glucosinolates are found to be effective against various types of cancer such as breast, lung and colon. Glucosinolates may breakdown to form isothiocyanates and/or nitriles in plants during processing, by the action of the endogenous enzyme myrosinase (thioglucoside glucohydrolase) or within the gastrointestinal tract by the action of commensal microflora.

Keywords: Brassica, glucosinolates, phytochemical, Thin layer chromatography (TLC), anticancer.

1. INTRODUCTION

Cruciferae family which is one of the largest families in the plant kingdom is rich in medicinal plants. It includes 338 genera and 3350 species that are distributed worldwide(1).Various studies indicate that consumption of large number of cruciferous vegetables (*e.g.,* broccoli, cabbage, kale, and Brussels sprouts) are associated with a reduced incidence of cancer (2) These contain various primary and secondary metabolites. The breakdown products of glucosinolates are (Indole-3- carbinol (I3C), di-indolylmethane (DIM). These degradation products have properties like antibacterial, anticancer and antifungal properties (3). The present study deals with phytochemical profiling of cabbage for presence of various phytochemicals. The extract was found to contain various secondary metabolites like tannins, flavonoids, sugars, alkaloids, phenols and anthocyanidin. The secondary metabolite glucosinolate, are the characteristic compounds of the crucifer family (4). These are group of compounds that are hydrolyzed either enzymatically with myrosinase or non-enzymatically to form primarily isothiocyanates and/or nitriles (4) Isothiocyanates were attributed to chemo-preventive activity, induce phase 2 detoxication enzymes, boost antioxidant status, and protect animals against chemically induced cancer. For further identification, of its degradation products, thin layer chromatography (TLC) was performed.

2. MATERIALS AND METHODS

2.1 Collection of Brassica oleracea (Cabbage)

Brassica Oleraceae 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 (5).

2.2 Extraction

The powder thus obtained was used for extraction purposes. Extraction was performed by following 2 methods:

2.2.1 Soxhlet Extraction: 8 gm of powder was extracted by soxhlet apparatus with 80% methanol (250ml) (5). After extraction, the solvent was evaporated and extracts were preserved at 4^0C. For phytochemical screening, extracts were dissolved in distilled water.

2.2.2 Cold Maceration: 10 gm of powdered cabbage was kept for 3 days on a rotary shaker. The supernatant obtained was utilized for phytochemical screening (5).

3. PHYTOCHEMICAL SCREENING

Phytochemical screening of extracts obtained by both the aforementioned mentioned methods was performed to profile various phytochemicals present in cabbage. The screening was done both qualitatively and quatitatively.

3.1 Qualitative Screening

3.1.1 Test for Tannins

About 2 ml of the aqueous extract was stirred with 2 ml of distilled water and few drops of $FeCl_3$ solution were added. The formation of a green precipitate was an indication for the presence of tannins (6)

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3.1.2 Test for phlobatannins

About 2 ml of aqueous extract was added to 2 ml of 1% HCl and the mixture was boiled. Deposition of a red precipitate was taken as an evidence for the presence of phlobatannins(6)

3.1.3 Test for flavonoids

To 1 ml of aqueous extract, was added 1 ml of 10% lead acetate solution. The formation of a yellow precipitate was taken as a positive test for flavonoids(6)

3.1.4 Test for phenols

Equal volumes (1 ml) of extract and Iron (III) chloride were mixed. A deep bluish green solution gave an indication of the presence of phenols(6)

3.1.5 Tests for carbohydrates

Molisch's test: 3 ml of the aqueous extract was added to 2 ml of Molisch's reagent and the resulting mixture shaken properly. 2 ml of concentrated H2SO4 was then poured carefully down the side of the test tube. A violet ring at the interphase indicates the presence of carbohydrate (7)

3.1.6 Tests for steroids

A red colour produced in the lower chloroform layer when 2 ml of organic extract was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid added indicates the presence of steroids (7).

3.1.7 Test for anthraquinones

0.5 g of the extract was shaken with 10 ml of benzene and filtered.10% of ammonia solution was added to the filtrate and the mixture was shaken. The formation of a pink, red or violet colour on the ammoniacal phase indicates the presence of anthraquinones (6).

3.2 Quantitative Estimation of Phytochemicals

3.2.1 Determination of total phenol

Total phenolic contents were determined by the Folin-CioCalteau method (Singleton et al. 1999). Each extract (obtained through soxhletion) (1 ml) was mixed with phenol reagent (1ml) and allowed to stand for 5 min at room temperature. Sodium carbonate solution (3ml, 2%) was added. Absorbance was measured after 2 hours incubation at 720 nm. A standard calibration curve was plotted using gallic acid. The total phenolic contents were expressed as mg of gallic acid equivalents (GAE)/g of plant material.

3.2.2 Determination of alkaloid

10ml of the extract (cold macerated) was taken into a 250 ml beaker and 10% acetic acid in ethanol (200ml) was added. Covered and allowed to stand for 4 h. This was filtered and the extract so obtained was concentrated on a hot plate to one-fourth of the original volume. Drop wise concentrated ammonium hydroxide was added to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium

hydroxide and then filtered. The residue obtained was alkaloidal in nature, which was dried and weighed (8)

3.2.3 Determination of Flavonoid

0.5ml of the plant sample was repeatedly extracted at room temperature with 100 ml of 80% aqueous methanol. The whole solution was filtered through whatman filter paper No 42 (125 mm). The filtrate was then transferred into a china dish and evaporated to dryness over a hot plate and weighed.

3.2.4 Determination of Saponin

10ml of cold macerated extract was dispersed in ethanol (200 ml, 20%) and kept over a hot water bath for 4 h with continuous stirring at about 55ºC. The mixture was filtered and the residue re-extracted with 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at 90ºC. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol extracts was washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample was dried in an oven. The saponin content was calculated in percentage.

3.2.5 Determination of anthocyanidin:

1 mL of cold macerated extract and catechin standard solution (50-300 mg/L), vanillin in methanol [2.5 mL, 1% (w/v)], and HCl in methanol [2.5 mL of 9.0 N] were added and incubated at 30°C for 20 min, the absorbance was recorded at 500 nm.

3.2.6 Determination of tannin:

1 mL of cold macerated extract was made upto 7.5 ml and then 0.5ml Folin-Denis reagent and 1 ml sodium carbonate was added. Again volume was made up to 10 ml using distilled water. The absorbance was recorded at 720 nm. A standard calibration curve was plotted using tannic acid (9)

4. THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was used to separate the glucosinolates present in cabbage i.e. glucosinolates. A thin layer of silica and plaster of paris was used in 4:1 ratio (10). The slurry thus obtained was poured on glass plate as a thin layer. The plates were air dried and kept for activation in a hot air oven at 120^0C (10). This served as a stationary phase. Two mobile phases were utilized 1) Butenol: acetic acid: water in 4:1:5 proportion (10) and 2) Butenol: pyridine: water in 6:4:2 proportion (11) The sample was applied on the plates. The plates with loaded samples (80µl) were kept in a closed chamber, previously saturated with the solvent (both the above mentioned solvents separately). The process was allowed to run and dried. The plates were sprayed with ammonical silver nitrate (1 gm $AgNO_3$ + ammonia 2.5 ml dilute to 1 litre) (12). The plates were kept in hot air oven for 12 min at 120^0C and then were observed for spots and Rf value were calculated.

5. RESULTS

5.1 Qualitative analysis of phytochemicals

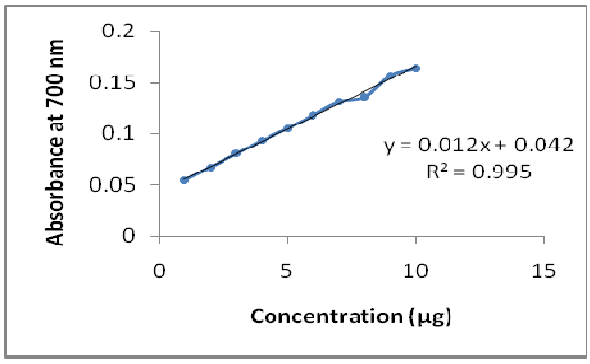
Table 1
Phytochemical profile of Brassica oleracea

TESTS	Soxhlet extraction	Cold extract
Sterols		
Salkowski's test	-	-
Liberman test	-	-
Alkaloids		
Dragendorf's reagent	++	++
Mayer's reagent	++	++
Wagner's test	++	++
Tannic acid test	++	++
Saponins		
Foam test	-	-
Flavonoids		
Flavonoids	+	+
Cardiac Glycosides		
Keller-Killiani test	+	+
Legal's test	+	+
Anthroquinones		
Bortranger's test	+	+
Tannin		
Ferric Chloride test	++	++
Lead acetate test	++	++
Potassium dichromate test	++	++
Gelatin solution test	++	++
Bromine water test	++	++
Phenols		
Ferric Chloride test	+++	++
Nitric acid test	+++	++
Phthalic acid test	+++	++
Proteins		
Biuret test	+++	+++
Xanthoproteic test	+++	+++
Amino acid test		
Ninhydrin test	++	++
Carbohydrates		
Molisch test	++	++
Fehling's test	++	++

+ indicates presence of respective phytochemical in trace but detectable amount.
++ denotes presence of respective phytochemical in good amount.
+++ indicates presence of respective phytochemical in high amount.
- indicates absence of respective phytochemical.

5.2 Quantitative estimation

5.2.1 Quantitative estimation of phenol:

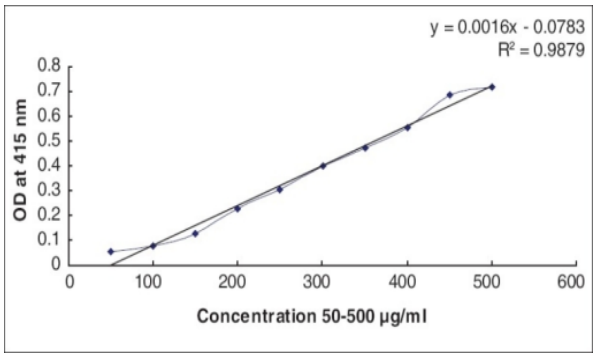


When a graph was plotted taking concentration on X axis and absorbance on Y axis and using gallic acid (0.05 g/ml) (13) as standard, the concentration of total phenols in *Brassica oleracea* was found to be 96.5µg in cold extract and 154.83 µg in soxhlet extract as gallic acid equivalents per gram of plant.

5.2.2 Quantitative estimation of Alkaloids

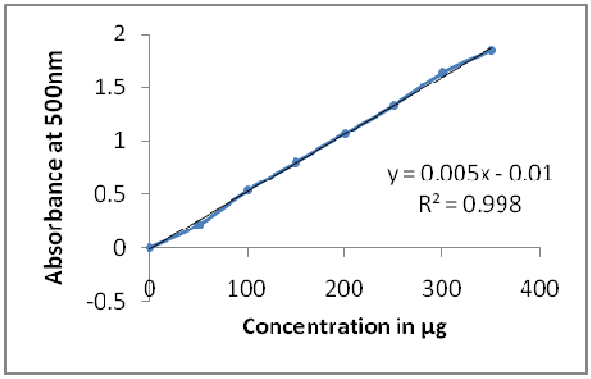
The dry weight of alkaloids estimated using standard protocol was found to be 0.891g in cold extract and 0.883 g in soxhlet extracted cabbage.

5.2.3 Quantitative estimation of Flavonoid



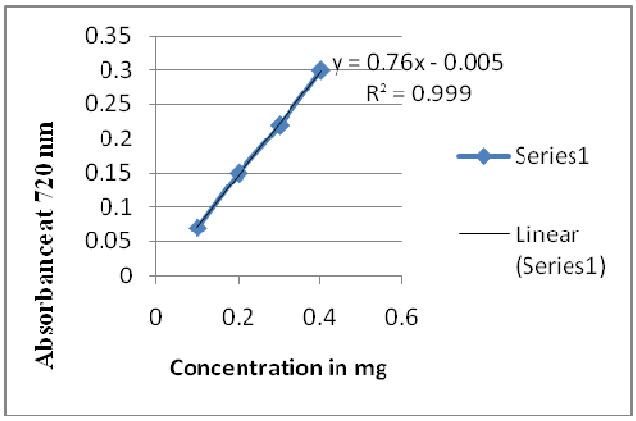
When a graph was plotted taking concentration on X axis and absorbance on Y axis and using quercetin (100-600 µg/ml) (13) as standard, the concentration of flavonoid in *Brassica oleracea* was found to be 1411µg in cold extract and 141.6 µg in soxhlet extract as quercetin equivalents per gram of plant material.

5.2.4 Quantitative estimation of Anthocyanidin



When a graph was plotted taking concentration on X axis and absorbance on Y axis using catechin as standard, the concentration of anthocyanidin in *Brassica oleracea* was found to be 283.8 µg in cold extract and 60.8 µg in soxhlet extract as catechin equivalents per gram of plant material.

5.2.5 Quantitative estimation of Tannin



Graph of concentration on X axis and absorbance on Y axis using tannic acid (0.01 mg/ml) as standard indicated the concentration of tannin in *Brassica oleracea* to be 0.25 mg in cold extract and 0.04 mg in soxhlet extact per gram of plant material.

5.3 Thin layer chromatography

Thin layer chromatography was performed to separate the glucosinolates (glucoraphanin glucobrassicin, glucoiberin, progoitrin, sinigrin, gluconapin, glucoerucin and neo-glucobrassicin) and its hydrolysis products (isothiocyanates, nitriles) from cabbage extract. Three extracts were used for TLC i.e. defatted soxhlet extract, defatted cold macerated extract and defatted fresh cabbage extract. For defatted Soxhlet and cold macerated extract, ground cabbage was defatted with diethyl-ether (in soxhlet, and on rotary shaker), then extracted with 80 % methanol and further utilized for TLC. For fresh cabbage extract, 30 gm of fresh cabbage with diethyl-ether was kept for 2 days followed by 80 % ethanol for 3 days on rotary shaker (same as for cold macerated extract). The supernatant obtained was utilized for TLC. After spraying and heating the TLC plates (as described above) the plates were checked for the development of spots. The spots obtained were then calculated for Rf values so that the compound can be identified. The Rf values were calculated by:

Retention Factor (Rf) vs. Solvent and Solute Polarity The Rf, or retention factor, of a compound is the ratio of the distance a compound travels up the TLC plate to the distance the solvent traveled. Rf = (distance migrated by spot) / (distance migrated by solvent) (14).

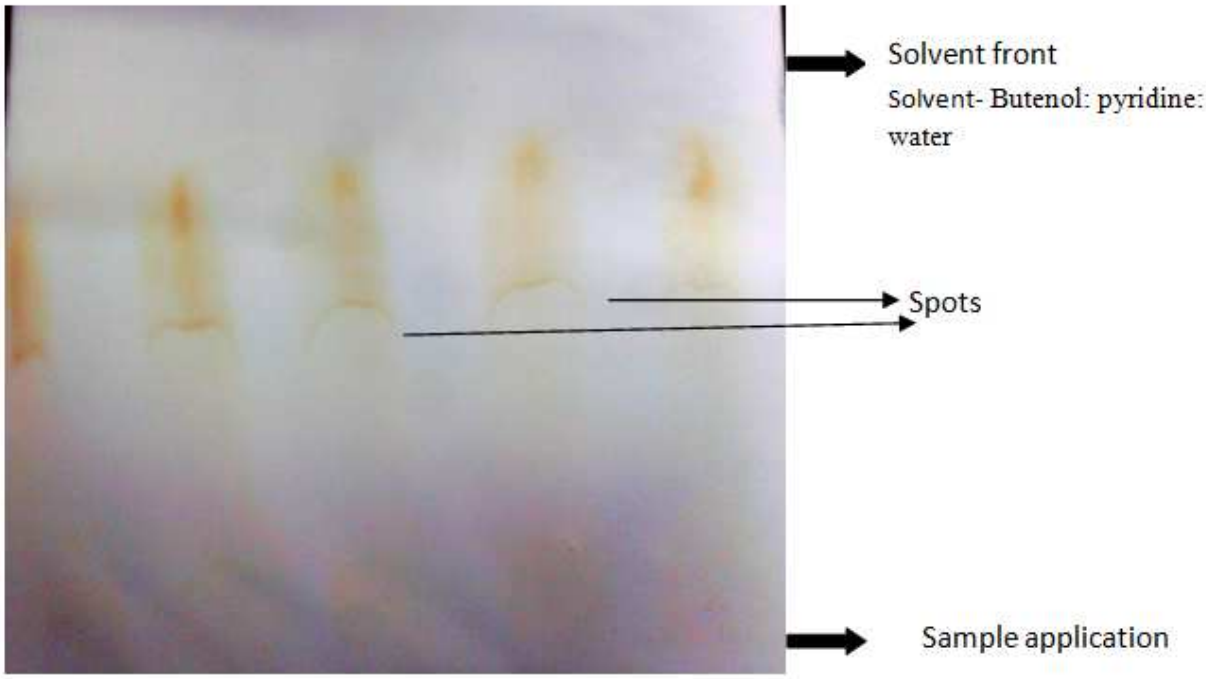


Figure 1
Chromatogram of Soxhlet extract

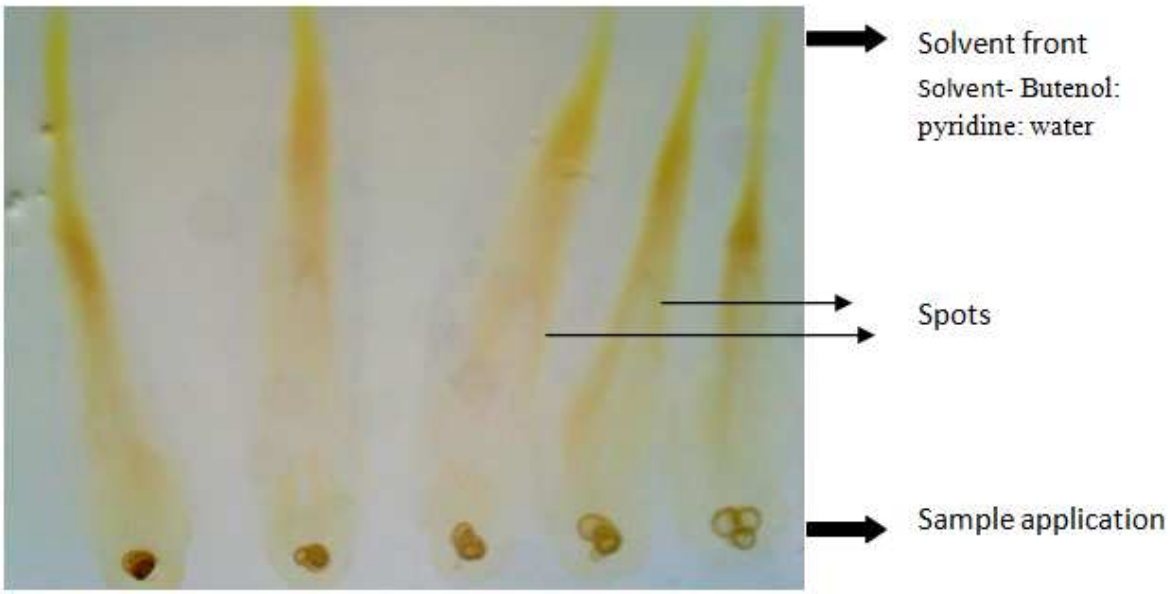


Figure 2
Chromatogram of cold macerated extract

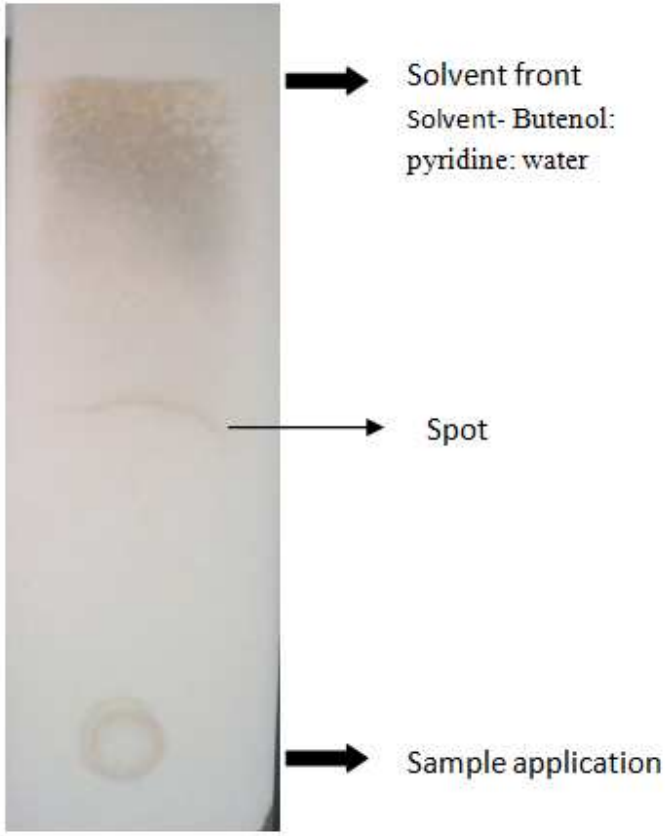


Figure 3
Chromatogram of Fresh cabbage

Extract	Phytochemical	*Rf*-values
Soxhlet Extract	Glucobrassicin	0.6
Cold extract	Glucobrassicin	0.6
Fresh cabbage extract	Glucobrasicin-1-sulphonate	0.3

Table 2
Detected glucosinolates in extracts and their Rf values

6. DISCUSSION

In the present study, phytochemical profiling of *Brassica olerecea* extract was performed. Results clearly indicated the presence of various phytochemicals like tannin, flavonoid, alkaloid, anthocyanidin and phenols. It is reported that cabbage has antibacterial, antifungal and anticancerous activity (5, 15). These activities may be attributed to various phytochemicals present in the extract. Tannin is reported to have antimicrobial activity and antibacterial activity. Alkaloids are

reported to act as antioxidant. TLC was performed to separate key component of cabbage extract i.e. glucosinolates, which are reported to have anticancerous activity. We have separated hydrolysis products of glucosinolates. This can be indicated from the known Rf values of the compounds (listed below). We have found the presence of glucobrassicin 0.6) and brassicin-1-sulphonate (0.3) which correspond to standard Rf values of glucobrassicin and brassicin-1 sulphonate (16). Also it has been noted that butenol: pyridine: water gave good results. Three extracts were used (soxhlet extract, cold macerated extract and fresh cabbage extract) so as to detect the presence of different glucosinolates in different extracts.

S.No	Glucosinolate standards	Rr values
1.	3-methylsulfinylpropyl GS	0.27
2.	3methylsulfonylpropyl GS	0.28
3.	Brassicin-1-sulphonate	0.3
4.	2-hydroxy-3-butenyl GS	0.39
5.	ally GS	0.43
6.	3-butenyl GS	0.46
7.	p-hydroxylbenzyl GS	0.50
8.	4-methylthiobutyl GS	0.51
9.	Glucobrassicin	0.6

This study further validates *Brassica oleracea* as an anticancer therapeutic plant. Presence of glucobrassicin and brassicin-1 sulphonate in the plant extract has been established with TLC. Also, the Rf values correspond to the compounds. This vegetable can be included in diet for its therapeutic benefit. Future work with respect to the toxicity studies and *invivo, invitro* anticancer activity of *Brassica oleracea* for breast cancer metastasis to bone is envisaged.

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