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COMPUTATIONAL AND BIOCHEMICAL ANALYSIS OF MYROSINASE FROM *BRASSICA OLERACEA*

Komal Talreja[1] and Archana Moon*[2]
[1&2] University Department of Biochemistry, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur -440033.
Email: moon.archana@gmail.com

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Abstract:

Glucosinolates are the characteristic secondary metabolites of the cruciferae family. Myrosinase is a β thioglycosidase enzyme, present in the specialized myrosin cells of leaves of *Brassica* family, which hydrolyse glucosinolates. The degradation products of glucosinolates are known to have antibacterial, antifungal and anticancer activities. The present study deals with the assay of sinigrin, a glucosinolate, in *Brassica oleracea* extract (hot soxhlet and cold macerated) and activity assay of myrosinase by a spectrophotometric method described by Charron *et a,l* in which sinigrin was used as a standard glucosinolate. Further breakdown of glucosinolates into isothiocyanates by myrosinase was determined by utilizing fresh cabbage extract as an enzyme source. Lastly, *insilico* studies were performed using Autodock Suite, version 1.5 6rC2, in which the interactions between ligand (glucosinolates) and protein (myrosinase) were estimated by Lamarckian genetic algorithm.

Keywords: Glucosinolates, myrosinase, *Brassica oleracea*, isothiocyanates, docking and Autodock.

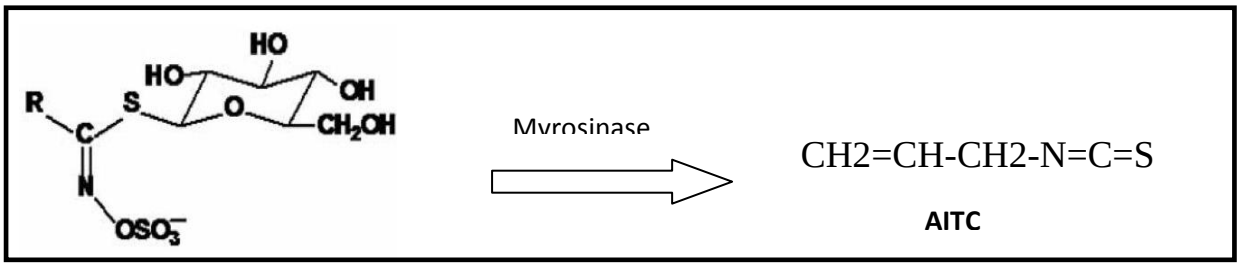
Introduction:

Glucosinolates are sulfur-containing secondary compounds characteristic of the *Cruciferae* family, containing the thioglucosidic bond, the sulfate anion, the glucosidic residue, and a side aglycon chain of aliphatic, aromatic, or heteroaromatic type (1). These are hydrolyzed enzymatically by myrosinase, which is present in the specialized cells known as myrosin cells (2). The enzyme is released from myrosin cells on cooking or chopping. The degradation products include isothiocyanates, nitriles thiocyanates, indoles and oxazolidinethiones. Isothiocyanates and indoles have been reported to have anticarcinogenic properties (3, 4). They block tumour initiation by modulating the activities of Phase I and Phase II biotransformation enzymes, and forcing tumor cells to opt for apoptosis (3).

Myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) is a glycopolypeptide containing various thiol groups and disulfide bridges), and a high percentage of carbohydrate (up to 22.5%), mostly hexoses. The enzyme consists of two identical subunits with a molecular weight of 71.7 kDa (5). Myrosinase catalyzes the cleavage of the *S*-glucose bond

by a acid/base-catalyzed reaction with the release of aglycon and the formation of the glycosyl enzyme intermediate (6). The myrosinase-glucosinolate system is present in various concentrations in all Brassicaceae leaves. When activated following tissue damage, it plays a defensive role against plant pests (6). In the present study, firstly the glucosinolate (sinigrin) was estimated in all the three extract (hot soxhlet, cold macerated). Once the concentration of sinigrin in extracts was ascertained, then hydrolysis of sinigrin into allyl isothiocyates was measured. This ultimately related to the activity of myrosinase, which was able to convert sinigrin into allyl isothiocyanate.

Further, myrosinase was docked with three glucosinolates viz; Glucobrassicin, Glucobrassicin 1 sulphonate and Sinigrin. These *in silico* studies were performed to detect the exact interaction between myrosinase and glucosinolates.



Glucosinolate

Materials and Method:

Brassica oleracea (cabbage) was used as an enzyme source for sinigrin estimation. Soxhlet, cold macerated extract and fresh cabbage extract were utilized for assaying the myrosinase activity.

Preparation of Cabbage Extract for sinigrin estimation:

Soxhlet Extraction: 8 gm of shade dried cabbage powder was extracted by a Soxhlet apparatus with 80% methanol (250ml) (2). After extraction, the solvent was evaporated and extracts were preserved at 4^0C. For phytochemical screening, extracts were dissolved in distilled

Cold Maceration: 10 gm of powdered cabbage was kept for 3 days on a rotary flask shaker. The supernatant obtained was utilized for phytochemical screening (2).

Preparation of Fresh cabbage Extract for myrosinase activity:

Fresh *Brassica oleraceae* was purchased from a local grocer.10% homogenate was prepared in buffer consisting 30 mM phosphate buffer (pH 7) and 1 mM EDTA. The homogenate was centrifuged at 5000 rpm for 10 min.The supernatant was used as enzyme source (1).

Sinigrin Estimation: A 5ml reaction mixture was prepared containing 50 µl of crude extract, 1.35 ml of 32.22 mM phosphate buffer with 1.07 mM EDTA and 100 µl of 37.50 mM sinigrin. Different concentrations of sinigrin were

used to plot the standard curve. Therefore, concentrations of the combined reaction mixture were 30 mM phosphate buffer, 1 mM EDTA and 2.5 mM sinigrin. The sinigrin content in hot soxhlet extract and cold extract was estimated. The absorbance was measured at 227nm (7).

Myrosinase Activity Assay:

Myrosinase (EC 3.2.3.1) activity assay was performed by a spectrophotometric method as described by *charron et al* (1) and was determined by decrease in absorbance at 227 nm. A 5ml reaction mixture was prepared that contained 50 µl of crude extract, 1.35 ml of 32.22 mM citrate/phosphate buffer with 1.07 mM EDTA and 100 µl of 37.50 mM sinigrin. Therefore, concentrations of the combined reaction mixture were 30 mM phosphate buffer, 1 mM EDTA and 2.5 mM sinigrin. A 1.5 ml reference mixture was prepared with distilled water instead of sinigrin (1). The decrease in absorbance was recorded to calculate myrosinase activity.

Estimation of allyl isothio cyanate:

The total isothiocyanate content of the fresh cabbage hydrolysate was determined by quantification of the cyclocondensation product between the isothiocyanates and 1,2-benzenedithiol. The cyclocondensation assay relies on the reaction of the carbon atom of the –N=C=S group of isothiocyanate molecule with thiol groups of 1,2-benzenedithiol to form a five membered 1,3-benzodithiole-2-thione and the corresponding amine (8). Briefly, in a 4 mL glass vial, each sample (0.25 mL) was mixed with 0.25 mL of 100 mmol/L potassium phosphate buffer (pH 8.5), 0.5 mL of 10 mmol/L 1,2-benzenedithiol in methanol to form a 1.0 mL reaction mixture (adjusted with water). The reaction mixture was incubated for 2 hours at 65 °C, and was then cooled to room temperature. The absorbance was measured at 365 nm. A standard curve was generated from measurements using varying amounts of pure allyl isothiocyanate to estimate the amount of cyclocondensation products (isothiocyanates) in test samples (8).

***In silico* studies:**

In silico studies were performed using Autodock 4 suite. Docking studies enables to evaluate the interaction between active site residues of the protein and ligands. The ligand binding efficiency of all the three ligands Glucobrassicin, Glucobrassicin 1 sulphonate and Sinigrin were tested towards myrosinase. The myrosinase structure was downloaded from PDB with PDB Id 1E4M (X ray diffraction structure, 1.2 A^0). The structure of ligands were downloaded from Pubchem (chemical structure data base) online portal and drawn in Marvin Sketch version 5.8.1. After docking, the results were analyzed on the basis of their binding energy and their interactions (9). Docking involves the use of scoring function to evaluate proper confirmation of ligand molecule with respect to binding energy. Protein and

ligands were imported, and then the grid was generated on the protein. Grid defines the active site (interaction points) of the enzyme. The coordinates (X axis, Y axis and Z axis coordinates) were defined for the active site where the ligand was to be docked. Autodock programme tries different conformations of the ligand in the active site(9).

Preparation of protein and Ligand

The structure of myrosinase was imported in Biovia Discovery Studio 2016 version 16.1.0.15350. The structure of protein was cleared (i.e. the extra groups which includes water molecules, ligand groups were removed) by deleting the heteroatoms present in the protein (10). This was saved as a PDB file. All the structure of ligands were downloaded from Pubchem and drawn in Marvin Sketch view version 5.8.1 and cleaned in 2D and 3D. This cleaned the 2 dimensional and 3 dimensional structure of the ligand. For docking, the protein structure in the PDB format and ligands in tripos-Mol format or PDB format were prepared (10).

Grid formation

Grid points generated the coordinates or interaction points where the ligand gets docked. 4 amino acids have been reported to form the active site of the myrosinase. These are ASN 265, Thr 267, ASP 268 and ALA 362. The grid box was generated at 60x60x60 A^0 to cover all the active site residues, and allowed the flexible rotation of ligands. The GA (genetic algorithm) and number of generation were set to 10 and 27000. The Lamarckian genetic algorithm was followed for ligand conformation. These all above parameters decided the different conformation of ligand in which the ligand will be docked. Other parameters viz; free energy (after docking is complete we get the value of free energy), rotatable bonds (number of rotatable bonds varies according to the ligand structure), number of torsions (10-11) etc were used as default (10).

PDB Structure of Myrosinase: 1E4M

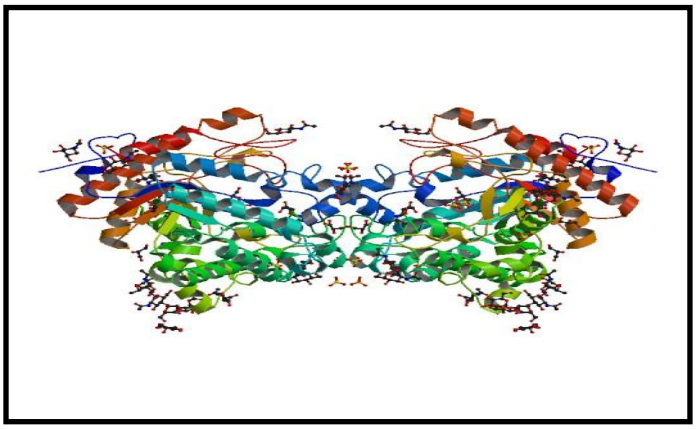


Fig1: x-ray diffraction with resolution of 1.2 Å

Protein structure cleared of heteroatoms using Biovia Discovery Studio version 16.1.0.15350.

Results:

Sinigrin estimation:

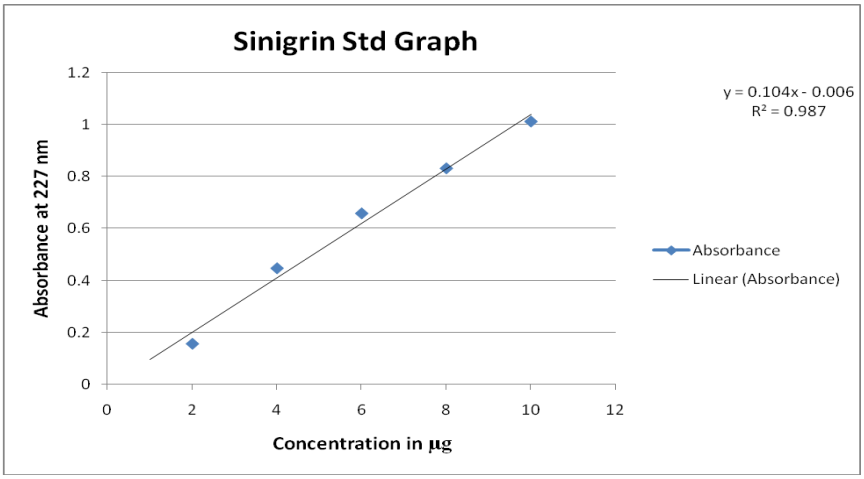


Fig 2: Standard graph of sinigrin.

Different concentratraions of standard sinigrin were used with reaction mixture containing phosphate buffer with EDTA and hot and cold extract of *Brassica oleracea.* Concentration of sinigrin in hot soxhlet extract was found to be 250 mg sinigrin/ 100 gm extract and 162 mg sinigrin/100 gm of extract in cold macerated extract. The supernatant obtained from cold extract was utilized for phytochemical screening and enzyme estimation (2).

Myrosinase activity was estimated by a spectrophotometric method. The same reaction mixture was utilized (Phosphate buffer with EDTA, sinigrin) and hot, cold and extract fresh cabbage extract was added (1). It was observed that myrosinase is found to be more active in fresh cabbage extract.

The decrease in absorbance is measured at 227 nm. Myrosinase activity in fresh cabbage extract was found to be **4.09 µmol of sinigrin degraded/min/mg of protein.**

The allyl isothiocyanate content of the fresh cabbage was determined by quantification of the cyclocondensation product between the isothiocyanates and 1,2-benzenedithiol. The reaction mixture consisted of sample, phosphate buffer and 1,2 benzenedithiol in methanol (8).

The absorbance was read at 365 nm. Allyl Isothiocyanate was found to be **2.5%** when 10 % fresh cabbage extract was used as an enzyme source.

Docking studies provide an insight into the interaction of protein with the ligand, type of interaction, amino acids involved in the interactions and binding energy.

Binding energy should ideally be negative. More negative the binding energy, better the binding affinity of ligand and the protein(10).

Table 1 shows the binding energy of different ligands with myrosinase.

Table a: Represents the Ligand Binding Energy with Myrosinase:

S.No	Ligands	Binding Energy
1.	Glucobrassicin	**-6.14**
2.	Glucobrassicin 1 Sulphonate	**-5.36**
3.	Sinigrin	**-4.94**

Table b: gives the interaction of ligand with Myrosinase i.e. hydrogen bond, amino acid involved in interaction and bond length. Three ligands were selected, glucobrassicin, glucobrassicin 1 sulphonate and sinigrin. These three ligands were selected Since glucobrassicin was found in hot soxhlet and cold macerated extract of *Brassica oleracea* and glucobrassicin 1 sulphonate was found in fresh extract of *Brassica oleracea*(2). Fresh extract was also used for myrosinase activity. Sinigrin was used as standard for glucosinolates and glucobrassicin, glucobrassicin 1 sulphonate was found in extracts. So all the three glucosinolates were used as ligands for myrosinase.

Table b: Represents Interaction of Ligand, type of interaction and bond length with myrosinase

S.No	Ligand	Interactions	Hydrogen Bond in A^0
1	Glucobrassicin	**LYS 387** **HIS 365** **ASN 9** **ASN 9**	**2.097** **2.046** **1.965** **1.852**
2.	Glucobrassicin 1 Sulphonate	**ASP 266** **ASN 9** **GLU 8**	**1.909** **1.96** **1.921**
3.	Sinigrin	**ASP 266** **PRO 263** **ASN 361**	**1.796** **1.862** 2.012

Myrosinase Interaction with Glucobrassicin: Docking studies with glucobrassicin showed 4 hydrogen bonds with myrosinase (figure 3). Two hydrogen bonds with ASN 9 (asparagine) (A), one hydrogen bond with His 365 (histidine) (B), one hydrogen bond with lys 387 (lysine) (C).

Figure I: Myrosinase interaction with Glucobrassicin.

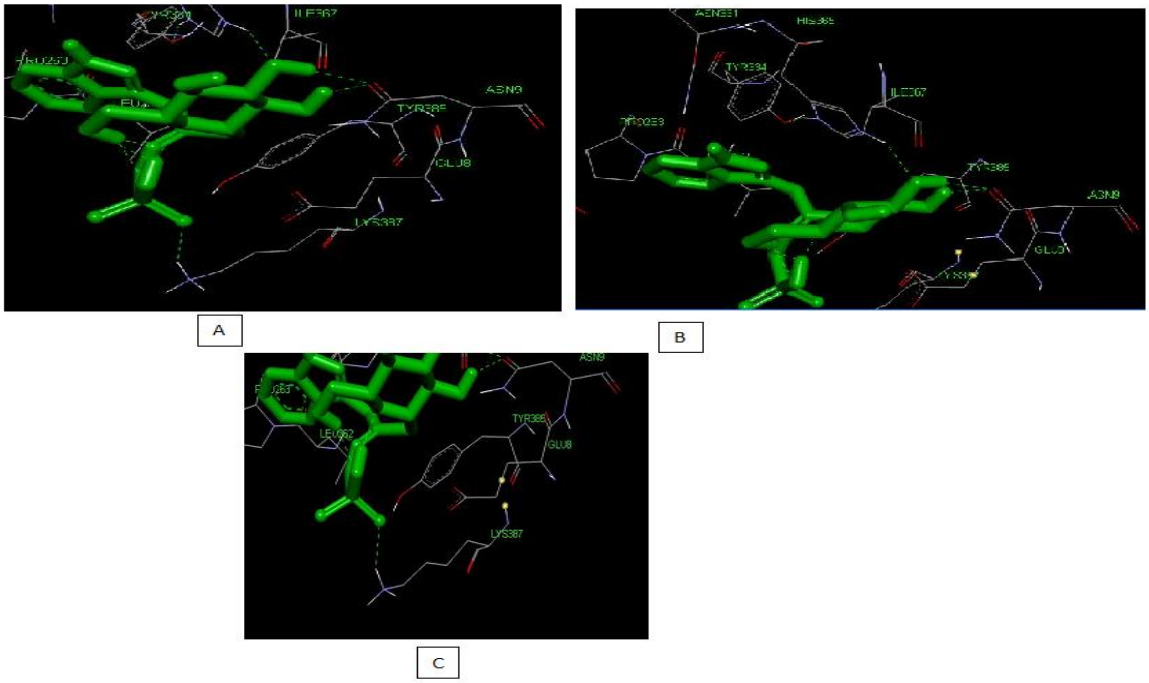


Fig I: Showing Myrosinase interaction with Glucobrassicin (A) 2 H-bond with ASN 9 (B) H-bond with HIS 365 (C) H-bond with LYS 387

Myrosinase Interaction with Glucobrassicin 1 Sulphonate

Glucobrassicin 1 sulphonate docked with myrosinase showed 3 hydrogen bond with myrosinase. One hydrogen bond with ASN 9 (asparagine) (A), one hydrogen bond with Asp 266 (Aspartate) (B), one hydrogen bond with Glu 8 (glutamate) (C).

Figure II: Showing Myrosinase interaction with Glucobrassicin 1 Sulphonate.

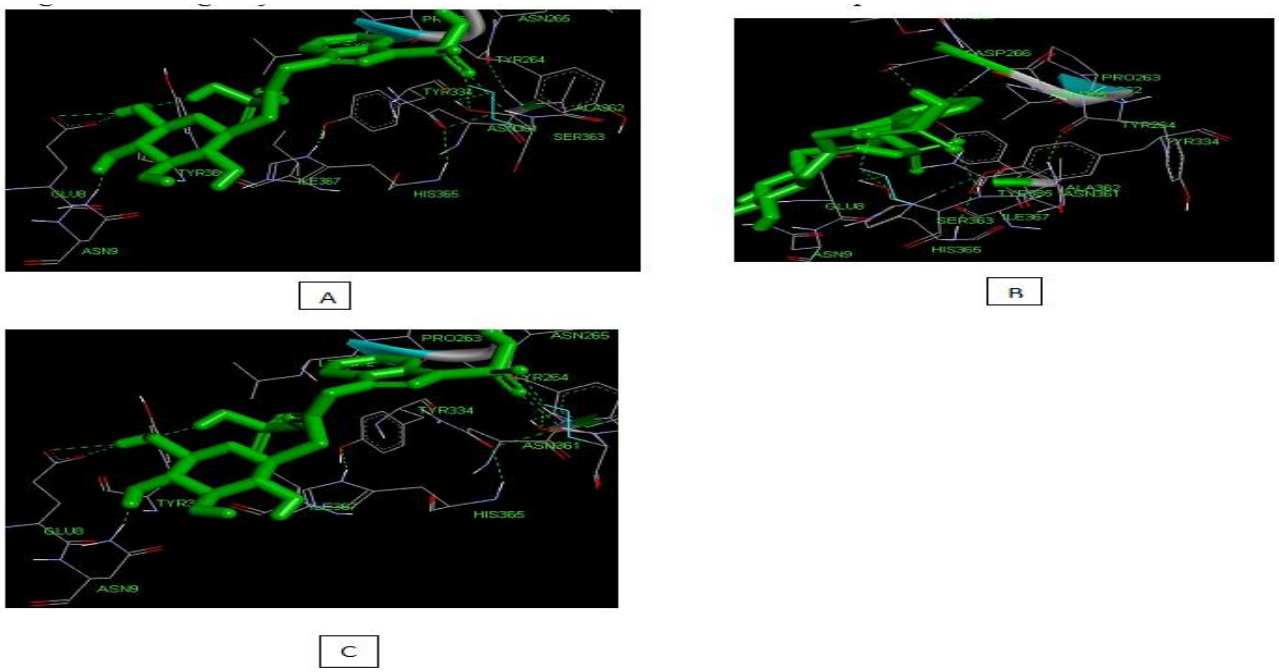


Fig II: Showing Myrosinase interaction with Glucobrassicin 1 Sulphonate (A) H-bond with ASN 9 (B) H-bond with ASP 266 (C) H-bond with GLU 8.

Myrosinase Interaction with Sinigrin

Docking studies showed that sinigrin interacts with 3 hydrogen bonds with myrosinase. One hydrogen bond with ASP 266 (asparagine) (A), one hydrogen bond with PRO 263 (proline) (B). One hydrogen bond with ASN 361 (C)

Figure III: Showing Myrosinase interaction with Sinigrin.

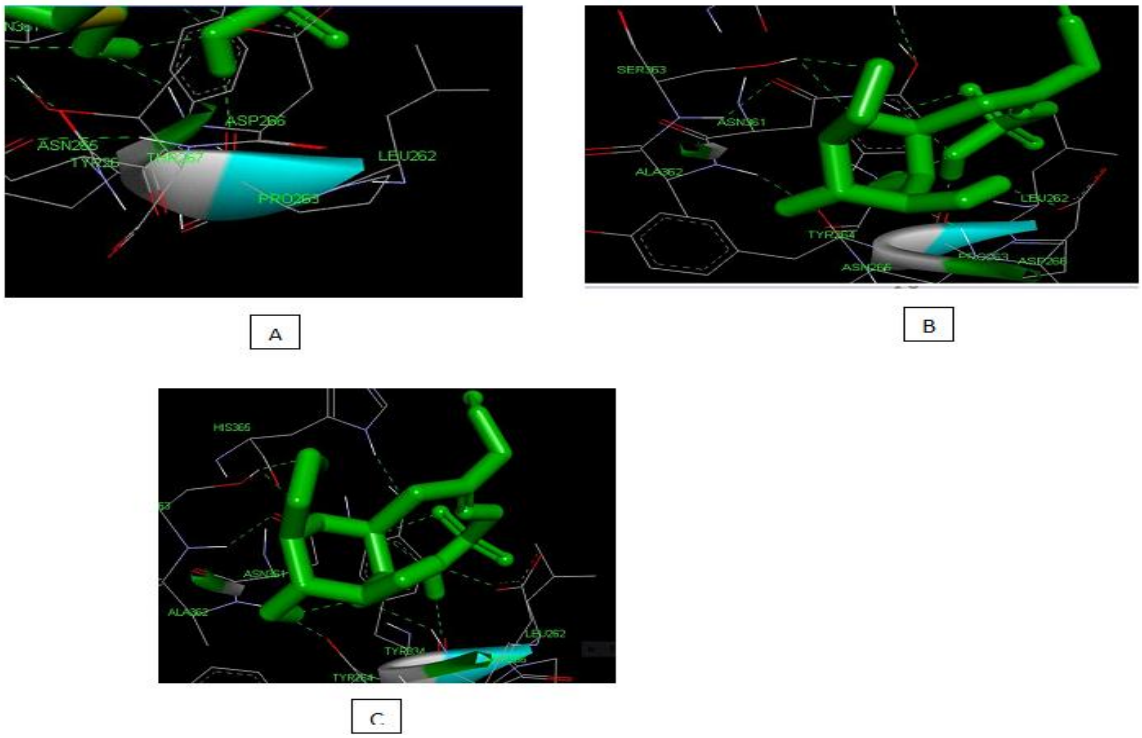


Fig III: Showing Myrosinase interaction with Sinigrin (A) H-bond with ASP 266 (B) H-bond with PRO 263 (C) H-bond with ASN 361.

Discussion:

Glucosinolates are characteristic secondary metabolites of the Cruciferae family. Glucosinolates are hydrolysed by myrosinase into its products (isothiocyanates and/or nitriles) (2). In the present study, sinigrin was estimated in cabbage extract (hot soxhlet, cold macerated). The concentration of sinigrin was measured in each of the extract. The activity of myrorinase was estimated with known concentrations of sinigrin (1). Myrosinase is an enzyme that is activated by chopping or cooking. It hydrolyzes glucosinolates and converts them into isothiocyanates or/and nitriles(12). Activity of myrosinase can be calculated in terms of amount of sinigrin hydrolyzed into allyl isothiocyanate. The hydrolysis product (allyl isothiocyanate) was also estimated for this purpose. Hence, clarity regarding quantity of glucosinolate converted to allyl isothiocyanate and enzymatic activity of myrosinase can be deduced. Our findings suggest that 250 mg sinigrin/ 100 gm extract was converted into 2.5% of allyl isothiocyanate with myrosinase activity of **4.09 µmol of sinigrin degraded/min/mg of protein.** This is the myrosinase activity, which can be used in treatment formulation, as the degradation products of myrosinase have anticancer, antifungal

and antibacterial activity (11). To harness the potential of *Brassica oleracea* (cabbage) extracts as anticancer, antibacterial and antifungal agents, this study gives an idea and insight into formulation development.

Insilico studies with myrosinase corroborate the potency of cabbage as an anticancer, antifungal and antibacterial agent via various hydrogen interactions of myrosinase with Glucobrassicin, Glucobrassicin 1 sulphonate and sinigrin. It showed 4 hydogen bonds with Glucobrassicin (LYS 387, HIS 365, 2 H bond ASN 9), 3 hydrogen bond with Glucobrassicin 1 sulphonate (ASP 266,ASN 9, GLU 8) and 3 hydrogen bond with sinigrin (ASP 266 ,PRO 263, ASN 361). But glucobrassicin has the lowest binding energy, it is the best substrate for myrosinase. For treatment with glucosinolates, glucobrassicin will be best as it has the lowest binding energy, will combine with myrosinase easily. Myrosinase is also present in gut microflora, even if myrosinase in cabbage gets inactivated while cooking, the gut microflora can act on glucosinolates and convert it into isothiocyates and/or nitriles (12).

Conclusion:

The present study demonstrates the amount of glucosinolate, sinigrin in cabbage extract. These compounds exhibit various properties viz; anticancer, antibacterial and antifungal. The extracts can be used as preventive measure against cancer after due ADMET testing, *invivo* and *invitro* studies. (11). Myrosinase enzyme assay, the enzyme which converts glucosinolates into active components was performed (13). Lastly the concentration of isothiocyanates, which is the major degradation product of glucosinolates was estimated. So, to harness the potential of *Brassica oleracea* (cabbage) extracts as anticancer, antibacterial and antifungal agents, this study gives an idea and insight into formulation development.

We can conclude that standard glucosinolate (sinigrin) in hot soxhlet extract 250 mg sinigrin/ 100 gm extract is converted into 2.5% allyl isothiocyanate with myrosinase activity **4.09 µmol of sinigrin degraded/min/mg of protein.**

Insilico results showed various hydrogen bonds with glucobrassin, gucobrassicin 1 sulphonate and sinigrin. 4 hydogen bonds with Glucobrassicin, 3 hydrogen bond with Glucobrassicin 1 sulphonate and 2 hydrogen bond with sinigrin. But the lowest binding energy is with Glucobrassicin (-6.14) (14). In conclusion, best interaction of myrosinase is with glucobrassicin. We had also reported the presence of glucobrassicin in hot soxhlet and cold macerated extract and glucobrassicin 1 sulphonate in fresh extract of *Brassica oleracea* by TLC (3). From the 120 types of reported glucosinolates, the best option would be to use glucobrassicin in treatment formulation as it has highest affinity towards myrosinase, as it easily gets converted into the active compound (11).

Acknowledgement:

University Research Project: Development of Murine model for breast cancer metastasis to bone. NO Dev/AH/2135 dated 16 November 2015

References:

1. Craig S Charron,1 Arnold M Saxton2 and Carl E Sams1, Relationship of climate and genotype to seasonal variation in the glucosinolate– myrosinase system. II. Myrosinase activity in ten cultivars of *Brassica oleracea* grown in fall and spring seasons, *Journal of the Science of Food and Agriculture, J Sci Food Agric* 85:682–690 (2005) DOI: 10.1002/jsfa.2031.
2. 1komal talreja and *2archana moon *Brassica oleracea*: phytochemical profiling in Search for anticancer compounds, International Journal of life science and pharma research, vol 4/ issue 4/oct-dec 2014, ISSN 2250-0480.
3. Komal Talreja and Archana Moon**Brassica oleracea*: a potent antioxidant therapeutic in health and diseases, International journal of pharmaceutical Sciences and Research, *IJPSR, 2015; Vol. 6(10): 4448-4452,* P-ISSN: 2320-5148.
4. Valéria Dal Práa , Natália Silva Jardima, Carolina Bolssoni Dolwitscha, Marcio Antônio Mazuttib, Carine Vianaa, Denise Bohrera, Paulo Cicero Nascimentoa, Leandro Machado de Carvalhoa, Marcelo Barreto da Silvac, Camilo Amaro de
5. Carvalhod, Marcelo Barcellos da Rosaa*A review of influence of environment and process parameters on glucosinolate-myrosinase system from *Brassica* Journal of Applied Pharmaceutical Science Vol. 3 (08), pp. 121-128, August, 2013,Available online at http://www.japsonline.com, DOI: 10.7324/JAPS.2013.3922
6. Renato Iori", Patrick Rollinb, Harald Streicher", Joachim Thiem", Sandro Palmieri"l*The myrosinase-glucosinolate interaction mechanism studied using some synthetic competitive inhibitors, , Federation of European Biochemical Societies, FEBS Letters 385 (1996) 87-90
7. roberta bernardi, michelina g. Finiguerra,‡ alessandro a. Rossi, and sandro palmieri*Isolation and Biochemical Characterization of a Basic Myrosinase from Ripe *Crambe abyssinica* Seeds, Highly Specific for *epi*-Progoitrin†, Journal of Agricultural and Food Chemistry, *J. Agric. Food Chem.* 2003, *51*, 2737-2744 10.1021/jf020796g CCC: $25.00 © 2003 American Chemical Society

8. M. Grazia Botti, Malcolm G. Taylor and Nigel P. Botting Studies on the Mechanism of Myrosinase Investigation of the effect of glycosyl acceptors on enzyme activity
9. Li Tang*, Joseph D. Paonessa, Yuesheng Zhang, Christine B. Ambrosone, and Susan E.McCann Total isothiocyanate yield from raw cruciferous vegetables commonly consumed in the United States, *J Funct Foods*. 2013 October 1; 5(4): 1996–2001. doi:10.1016/j.jff.2013.07.011.
10. pallavi sahare[11] , archana moon *In silico* modelling of β-lactam resistant *enterococcus faecalis* pbp4 and its interactions with various phyto-ligands, International Journal of Pharmacy and Pharmaceutical Sciences, Vol 8, Issue 7, 2016
11. P. Sahare and A. Moon * *In-silico* docking studies of phyto-ligands against *e. Coli* pbp3: approachTowards novel antibacterial therapeutic agent *IJPSR, 2016; Vol. 7(9): 3703-3711. International Journal of Pharmaceutical Sciences and Research*
12. K. Talreja and A. Moon*,Effect of nutrients on liver marker enzymes of wistar rats International journal of pharmaceutical sciences and research, IJPSR (2016), Vol. 7, Issue 5
13. Theresa A. Shapiro,2 Jed W. Fahey, Kristina L. Wade,Katherine K. Stephenson, and Paul Talalay Chemoprotective Glucosinolates and Isothiocyanates of Broccoli Sprouts: Metabolism and Excretion in Humans1*Vol. 10, 501–508, May 2001* Cancer Epidemiology, Biomarkers & Prevention
14. Anna Piekarska, Tadeusz Pilipczuk, Barbara Kusznierewicz, Jace Namieśnik, Agnieszka Bartoszek The influence of cultivation conditions on the myrosinase activity And glucosinolate content in white cabbage Faculty of Chemistry, Gdansk University of Technology.
15. Pallavi sahare, archana moon*, tanvee pardeshi and sameer chpudhary The analysis of inhibitory activity of phyto-ligands against tem B-lactamase from multidrug resistant *escherichia coli archana moon* et al. International journal of pharmacy & technology.*