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Use of dietary phytochemicals in gastric cancer and ulcer

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Abstract

Gastric cancer is one of the main cause of cancer related death among both sexes across the globe. Conventional cancer treatments which include chemotherapy and radiotherapy often have harmful and undesirable side effects and therefore, nowadays focus is on the use of plant and spices extract to treating cancer with minimum side effects. The aim of our present study is to evaluate the anticancer properties of two Indian spices, garlic and yellow mustard, both individually and in combination, at specific concentrations with respect to certain antioxidant enzymes like Glutathione-s-transferase (GST) and Superoxide dismutase (SOD), cellular signalling enzyme like Nitric oxide synthase (NOS) and intracellular antioxidant, Glutathione (GSH), *in vitro* condition, against gastric cancer and ulcer tissue and normal healthy individuals respectively. Garlic and mustard can lower the risk of ulcerous growth, cancer and tumour formation in an individual by reducing the effect of oxidative stress through increase in SOD and decrease in NOS activity and also eliminate carcinogenic compounds through enhancement of GST activity and GSH concentration. Thus, the present study revealed that the extracts of garlic and mustard can act synergistically and individually, at appropriate concentration, on gastric cancer and ulcer respectively.

Keywords: Cancer, Garlic, Mustard, Superoxide dismutase, Glutathione S- transferase, Nitric oxide synthase, Glutathione.

1. Introduction

Stomach is one of the 5 leading sites of cancer in both sexes, according to World Health Organization and thus gastric cancer remains the fourth-most common cancer ^[1] and the second most common cause of cancer death world-wide ^[2]. The symptoms and signs of gastric cancer are often reported late when the disease is already in advanced stage and 5-year survival rate is less than 30% in many countries ^[2].

Interest in the benefits of bioactive compounds from plant source has origins in ancient past as they are used for treatment of diseases and maintenance of health. In today's time, when conventional therapy finds itself insufficient in various diseases, many people are looking for alternative medicines to help manage or prevent the onset of chronic disease, improve cognitive function, boost overall general well-being, and increase longevity. Epidemiologically and laboratory based studies indicate that consumption of fruits and vegetables ^[3] and inclusion of herbs and spices in our daily diet can reduce the risk of gastric cancer ^[4, 5].

Our study aims to highlight the potency of two Indian spices, garlic (*Allium sativum*) and mustard seeds (*Brassica juncea*), against gastric cancer, *in vitro* condition, through modulation of antioxidant and cellular signalling enzymes and by maintenance of redox equilibrium through increase in glutathione level. Garlic and mustard individually are known to reduce the risk of gastric cancer through induction of apoptosis, stimulation of phase II drug metabolizing enzymes and through anti-angiogenesis ^[6, 7] but their synergistic effect at appropriate dosage with respect to their antioxidant properties against gastric cancer have not been studied yet, so this study also helps in finding the additive effect of these two spices against the said disease, *in vitro*. As gastric ulcer is positively correlated with the risk of developing gastric cancer later in life ^[8], if it is left untreated, so we included ulcers of the stomach as well in our study.

Phase II drug metabolizing enzyme, Glutathione S-Transferase (GST) distributed widely in tissues such as liver, brain, intestine etc. can promote detoxification of electrophilic xenobiotics including carcinogenic elements by catalysing their conjugation to glutathione, making them less harmful and more soluble which are further metabolized and excreted from the body [9, 10]. Organosulfur compounds from garlic and allyl isothiocyanate from mustard have been known to increase the activity of GST in animal model [11, 12]. Superoxide dismutase (SOD) provide defence against the damaging activities of the superoxide radical by catalysing their dismutation into oxygen and hydrogen peroxide which further decomposes into water and molecular oxygen by the action of catalase and thus act against cancer [13]. SOD activity increases in response to bioactive compounds from garlic and mustard [14, 15].

Nitric oxide synthases (NOS) produces nitric oxide (NO) from L-arginine. Increased NOS expression was observed in lung and colon cancer and there have been reports that suggest NO acts as a carcinogen. NO causes DNA damage in the course of nitration, nitrosation and deamination. It also inhibits DNA ligase activity resulting in the accumulation of DNA breaks. Elevated NO production enhances the growth of some tumours through the suppression of anti-tumour immune responses [16, 17]. Inducible NOS (iNOS) activity have been shown to be reduced on treatment with organosulfur compounds from garlic and mustard in different conditions [18-20].

The detoxification potential of an anti-oxidant, Glutathione (GSH), has made the clinical studies of its tissue level possible in relation to cancer therapy. To some extent, the reduction-oxidation balance in tissue is managed by the concentrations of reduced glutathione and its oxidized disulfide counterpart which in turn effects gene expression, cellular differentiation, proliferation and apoptosis. Chemotherapeutic agents and radiation treatments induce some form of oxidative stress in the cancer cell and alter this redox balance. At this point GSH may play a key role in maintaining the redox state [21]. Bioactive principles of garlic and allyl isothiocyanate present in mustard helps in elevating the concentration of GSH in experimental model and different cell lines [22, 23].

For conformation of the disease, histological study and methylation level determination of the biopsy tissues were also conducted.

The investigation was approved by the Institutional Ethics Committee of IPGME&R, Kolkata (Registration No. ECR/35/Inst/WB/2013), for use of tissue specimen already obtained beforehand from subjects at upper GI endoscopy as part of their treatment plan and not just collected specifically for the purpose of the study.

2. Materials & Methods

2.1 Chemicals

1-chloro-2,4-dinitrobenzene [CDNB], Reduced Glutathione [GSH], 5,5'-dithiobis-(2-nitrobenzoic acid) [DTNB], phenyl methane sulfonyl fluoride [PMSF] were obtained from Sisco Research Laboratory Pvt. Ltd. 2-mercaptoethanol, Hepes buffer, Eosin and Haematoxylin staining reagents were purchased from HIMEDIA. Arginine monochloride, and potassium ferricyanide were purchased from Loba Chemie Pvt. Ltd. and Qualigens Fine Chemicals respectively. Pyrogallol was obtained from BDH chemicals. Msp I and Hpa II restriction enzymes pair were obtained from Genetix.

2.2 Preparation of garlic and mustard extracts

Garlic and yellow mustard seeds were obtained locally and fresh crude aqueous extracts of the spices were prepared utilizing the established protocol [24]. The concentrations of garlic and mustard used for the study were 800 mg/ml and 100 mg/ml respectively.

2.3 Preparation of tissue homogenates

Biopsy tissues from gastric cancer and ulcer patients and normal healthy individuals were collected in phosphate buffered saline (PBS) under ice cold condition from SSKM hospital, Kolkata. Some portion from each samples were utilized for histopathological and methylation level study. Homogenates of the tissue were prepared as per the protocol [24].

2.4 Preparation of experimental homogenates

The homogenate was fractioned as per the following groups for analysis [24-26].

For Normal tissue samples:-i} N1 (Control) ii} N2 (Tissue with garlic extract, 1:1 ratio) iii} N3 (Tissue with mustard extract, 1:1 ratio) iv} N4 (Tissue with both garlic and mustard extract, 1:1:1 ratio). Similarly, ulcer tissue and cancer tissue were grouped from U1 to U4 and C1 to C4 respectively for the experiments.

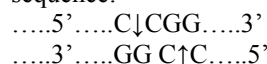
Analysis was done after incubating the samples for 3 hours. At the end of the incubation period the samples were assayed for the estimation of GST, SOD, NOS and GSH level in the tissues.

2.5 Histopathological study

Histopathology is the 'Gold Standard' for vast majority of diagnoses because most of the diseases can be or are defined by microscopic features and be compared with the macroscopic diagnosis for better assessment. Histological study was conducted using the standard technique of Haematoxylin and Eosin staining [27, 28].

2.6 Methylation level determination

DNA from the samples were isolated using the standardized protocol [29]. For restriction analysis, an enzyme pair of Msp I and Hpa II were used. Both enzymes cut DNA in the sequence:



Msp I and Hpa II are dissimilar in their sensitivity to DNA methylation. Msp I cut both outer and inner methylated cytosines while Hpa II cut only outer cytosines in the DNA sequence. On this basis, we determined the level of methylated fragments of genomic DNA from cancer and ulcer tissues and compared it with the control. For the experiment we followed the protocol of Shaden Muawia Hanafy *et al.* [30] with slight modifications. Purity of all the DNA samples were in the range of 1.7 – 1.9 and concentration of the DNA used were 1mg/ml.

2.7 Biochemical analysis

GST activity was assayed spectrophotometrically using reduced GSH and CDNB as substrate and measuring the increase in the concentration of conjugated product over time at 340 nm [31]. SOD activity was measured by means of Inhibition of Pyrogallol Autoxidation at 420 nm [32]. NOS assay was based on the conversion of arginine to nitric oxide and citrulline, which was conducted at 420 nm, with slight

modifications [33]. For GSH activity determination, the method was based on the reaction between DTNB (Ellman's reagent) and GSH, the product of which possesses its absorption maximum at 412 nm [34]. The protein contents of the samples were quantified by standard Folin-Ciocalteu method. The absorbance was measured at 660 nm.

2.8 Statistical Analysis

The data were analysed using Standard Deviation formulae. The results were expressed as mean ± S.D of 10 samples for each group.

3. Results and Discussion

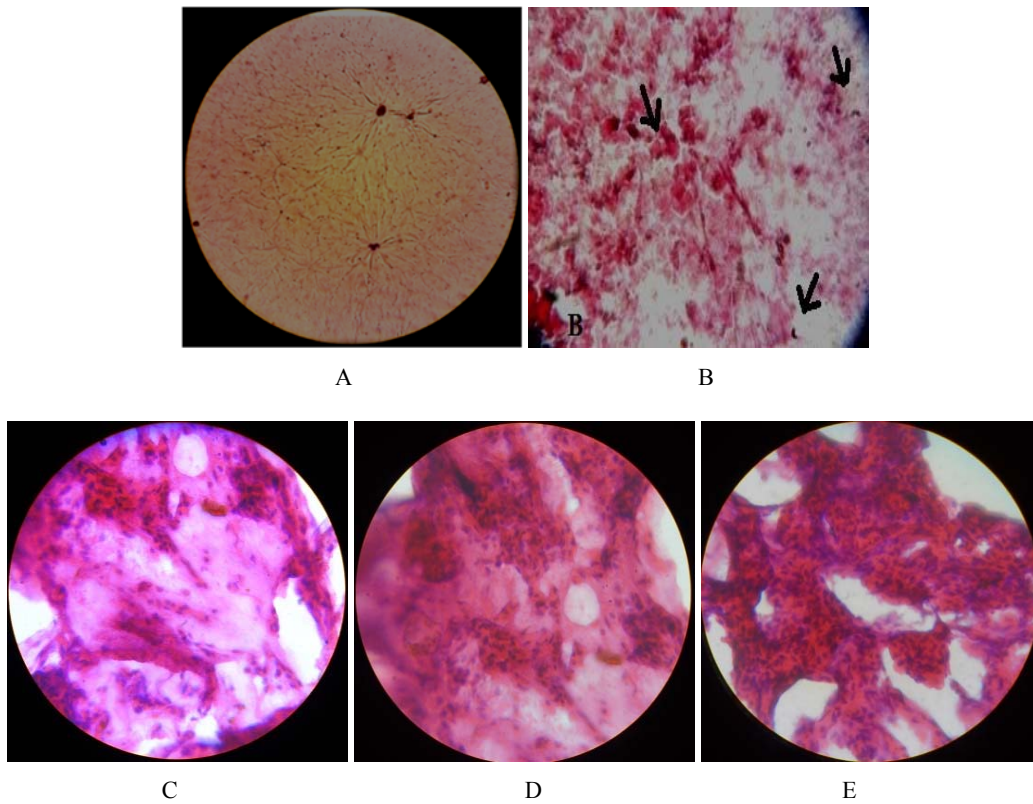


Fig 1: A – normal healthy individual gastric tissue stained by H&E technique, viewed under 40X ; B – gastric ulcer tissue (40X) ; C, D, E – gastric cancer tissue (100X)

3.1 Staining result: In H&E staining (Fig 1), haematoxylin, a basic dye colors nuclei of cells blue while eosin, an acidic stain colors eosinophilic structures in the cytoplasm pink and red. Gastric cancer tissues were characterized by extracellular mucinous pools, as a result of production of mucin, a glycosylated protein whose over expression is associated with many types of cancer [35]. The tumor cells

also showed marked tendency to 'basophilia', that is uptake of deep blue colouration of the structures under staining condition. Cancer tissue also showed pleomorphic nuclei (variability in size, and shape of the nuclei) with many cases showing considerable mitotic activity of the nuclei, in comparison to normal tissue which did not show any of such characteristics [36].

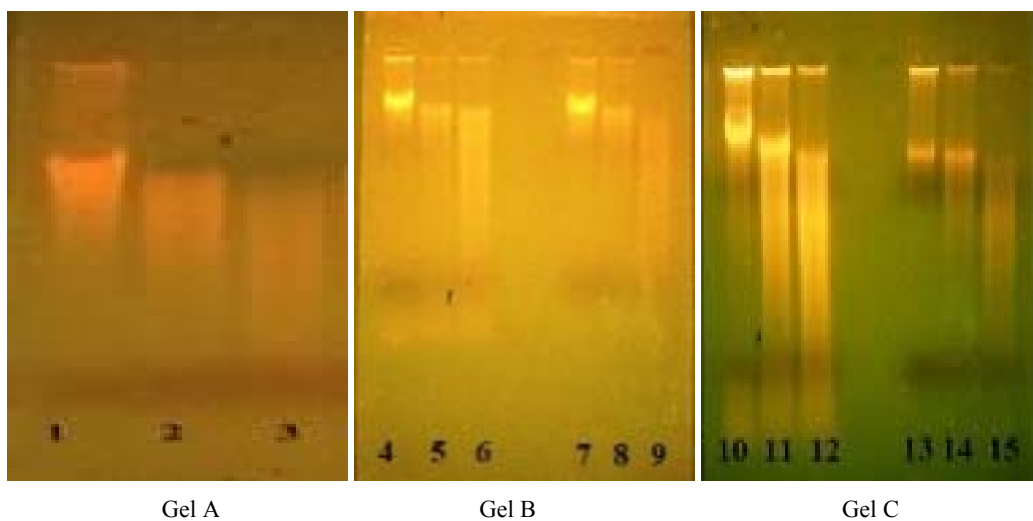


Fig 2: Lane 1, 2 and 3- DNA from normal sample. Lane 4, 5, 6, and 7, 8, 9 - DNA from cancer and ulcer patients respectively. Lane 10, 11, 12 and 13, 14 15- DNA from cancer patient and normal region of the same diseased patient respectively. 1, 4, 7, 10, 13- undigested, 2, 5, 8, 11, 14- digested with Hpa II and 3, 6, 9, 12, 15- digested with Msp I for 1 hour for all the cases..

3.2 Methylation study result: DNA methylation is a significant modification, typically occurring at 5³-CpG (Cytosine-phosphate-Guanine sites). In mammals, nearly 60% of promoters lie within this (5³-CpG) region and CpG methylation and transcriptional activity has an inverse relationship. There are evidences that cancer cells infringe this mechanism and use it to their benefit by inactivating tumour suppressor genes leading to cancer progression [30]. In our study (Fig 2), we could observe the difference in banding pattern between sample from normal region (lane 10, 11, 12) and from growth region (lane 13, 14, 15) of the same cancer patient (Gel C). There was less digestion of the DNA obtained from the tumorous growth region of the

cancer patient, by the restriction enzyme Hpa II as compared to samples from normal region of the same patient, which indicated hyper methylation of DNA sequences (CCGG) at CpG Island. Also when compared to cases from healthy individuals (Gel A), cancer cases again showed less digestion of the DNA whereas the DNA from normal individual were significantly digested by Hpa II and Msp I enzyme pair again indicating noteworthy differences in the methylation level of DNA from normal and cancer cases respectively. There were no comparable differences in the methylation level of cancer and ulcer patients (Gel B) or normal and ulcer patients respectively.

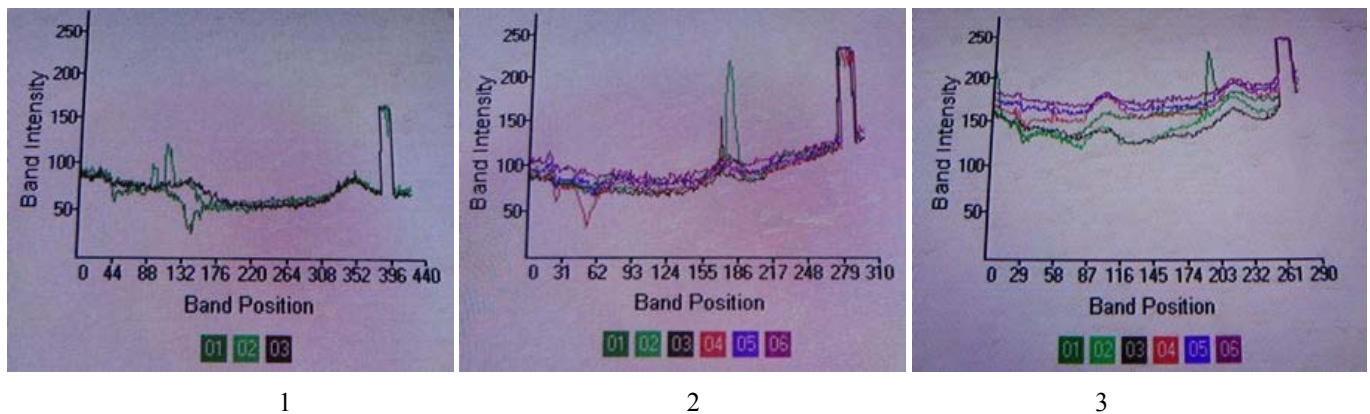


Fig 3: densitometer scan of Gel A – 1, Gel B – 2, Gel C – 3. On densitometric scans, curves of non-digested DNA and DNA treated by the restriction enzymes had different shapes for each sample.

3.3 Glutathione-s-transferase: GST genes are down regulated in many types of human malignancies, especially the gene GSTP1 is silenced, mediated by the promoter DNA hyper methylation [37]. The activity of GST was found to be the highest in normal (N1) tissues (0.18±0.03 U/mg) as compared to ulcer, U1 (0.122±0.009 U/mg) and cancer, C1 (0.024±0.005 U/mg) samples, which indicates that down regulation of GST gene may have occurred in ulcer and malignant patients but when aqueous garlic and mustard extracts were used against all the three types of tissues separately, at specified concentrations, then GST activity

increased significantly in each samples indicating that garlic or mustard might help in the reversal of hyper methylation and upregulate the activity of GST enzymes, thus helping in the detoxification process and protect against malignancy. Garlic and mustard in combination were found to increase the activity of GST in normal, N4(0.44±0.07 U/mg), ulcer, U4 (0.219±0.020 U/mg) and cancer, C4(0.095±0.008 U/mg) tissues, the most, in vitro condition, as compared to their individual effect, suggesting that these two spices can act synergistically in elevating the enzyme’s activity.

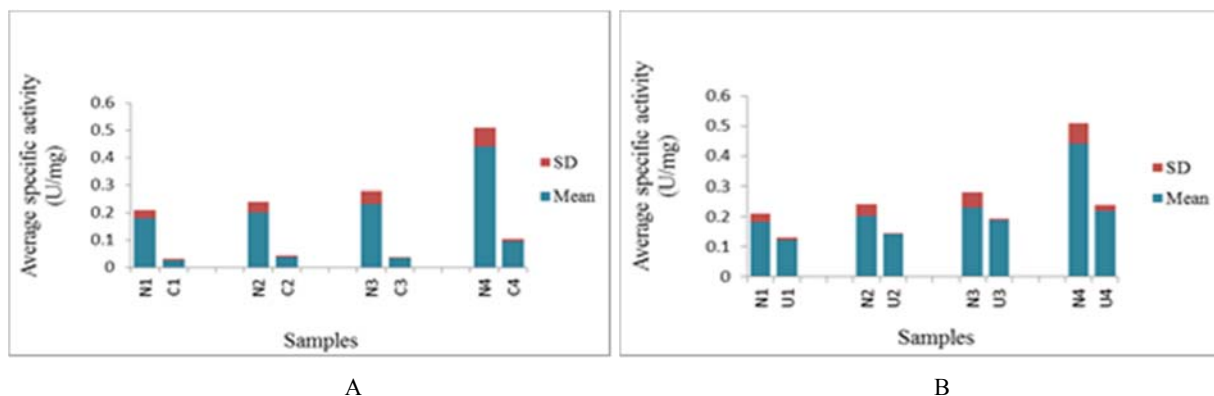


Fig 4: Average (Mean) specific activity of GST enzyme in tissue samples (A- cancer compared with normal; B- ulcer compared with normal) at 340 nm.

3.4 Superoxide dismutase: Activity of SOD, a tumour suppressor enzyme, was effectively increased in normal, ulcer and cancerous tissue respectively, on application of crude garlic and mustard extracts but it was mustard alone, at 100 mg/ml, which elevated SOD activity the most, both in ulcer, U3 (98.62±6.08 U/mg) and cancerous tissue, C3

(10.96±0.68 U/mg). The combined extract of both the spices could increase the SOD activity, the most, only in normal tissue, N4 (6.93±0.49 U/mg), so it can be inferred that mustard alone is more effective than garlic or the combined extract in combating the oxidative stress and thus act in protection, by enhancement of superoxide dismutase action.

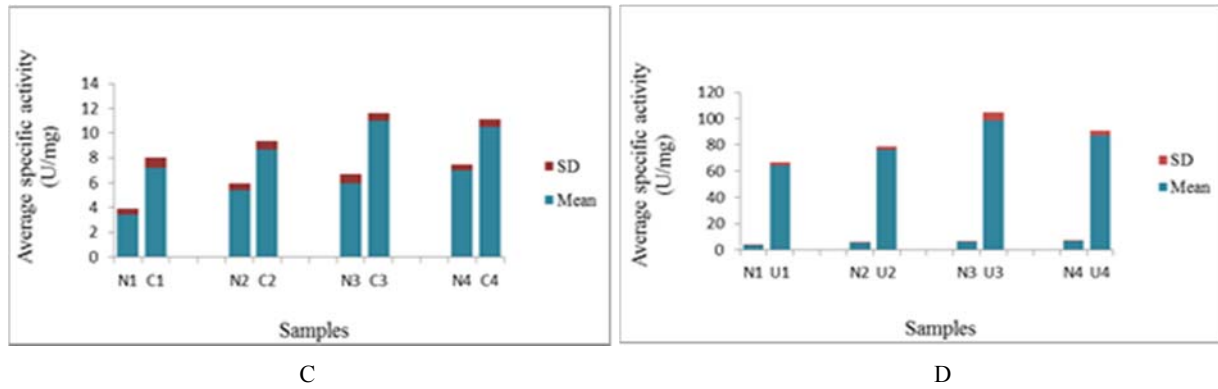


Fig 5: Average (Mean) specific activity of SOD enzyme in tissue samples (C- cancer compared with normal; D- ulcer compared with normal) at 420 nm.

3.5 Nitric oxide synthase: NOS activity was found to be higher in ulcer, U1 (122.99 ± 2.44 U/mg) and cancer, C1 (10.08 ± 0.59 U/mg) tissues as compared to normal, N1 (3.87 ± 0.21 U/mg) with ulcer showing highest expression of NOS. These observations suggest altered biochemical activity of the enzyme and indicate that it can as well act as a potential biomarker in gastric ulcer and cancer, as over expression of NOS has already been implicated in number of cancer types. Combined extract of garlic and mustard

could effectively decrease NOS activity in normal, N4 (2.01 ± 0.07 U/mg) as well as in cancer, C4 (6.78 ± 0.48 U/mg), the most while garlic alone at 800 mg/ml, reduced NOS activity in ulcer tissue, U2 (93.89 ± 1.78 U/mg). These results suggest that garlic alone or in combination with mustard can significantly reduce the activity of NOS enzyme, whose enhanced activity could lead to progression of gastric ulcer and cancer.

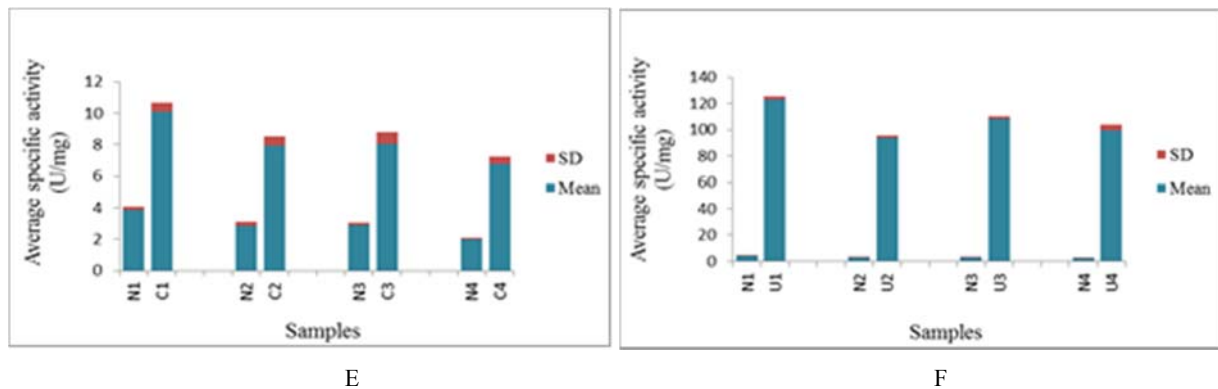


Fig 6: Average (Mean) specific activity of NOS enzyme in tissue samples (E- cancer compared with normal; F- ulcer compared with normal) at 420 nm.

3.6 Glutathione: Glutathione concentration in ulcer tissue, U1 (0.303 ± 0.03 U/mg) was found to be much higher as compared to normal, N1 (0.021 ± 0.003 U/mg) and cancer, C1 (0.006 ± 0.002 U/mg) sample, which had the lowest concentration of total glutathione but crude aqueous extract of garlic and mustard in combination helped in elevating the concentration of GSH in all the different samples, that is

normal, N4 (0.056 ± 0.006 U/mg) ; ulcer, U4 (1.605 ± 0.07 U/mg) and cancer, C4 (0.075 ± 0.020 U/mg) which proved their combined and synergistic efficacy in the detoxification process and maintenance of the redox balance in various conditions and thus may help in prevention of ulcerous or cancerous condition.

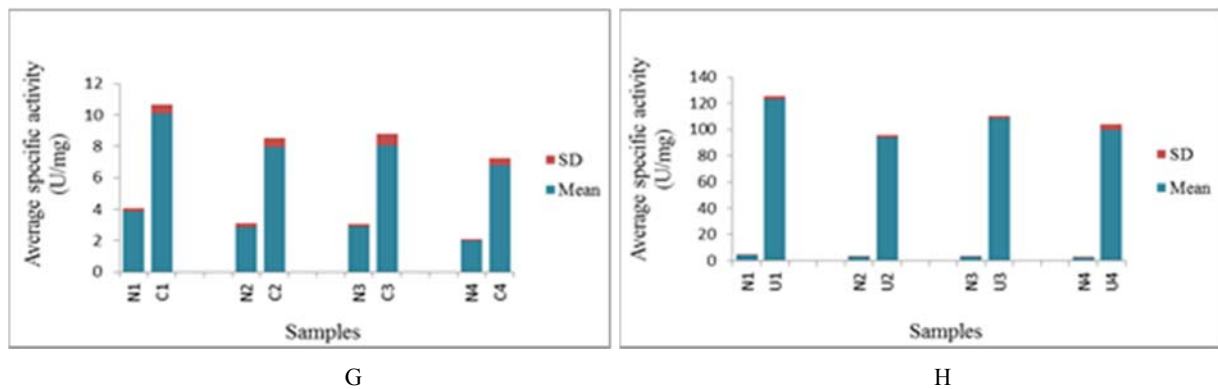


Fig 7: Average (Mean) Glutathione concentration in tissue samples (G- cancer compared with normal; H- ulcer compared with normal) at 412 nm.

Table 1: Normal tissue samples:-i} N1 (Control) ii} N2 (Tissue with garlic extract) iii} N3 (Tissue with mustard extract) iv} N4 (Tissue with both garlic and mustard extract). Similarly, ulcer tissue and cancer tissue were grouped from U1 to U4 and C1 to C4 respectively for the experiments.

Samples	Average specific activities of the biochemical enzymes (U/mg) Mean±SD (P value in bracket)			Avg GSH conc. (U/mg)
	SOD	NOS	GST	GSH
N1	3.44±0.45	3.87±0.21	0.18±0.03	0.021±0.003
U1	63.83±2.65	122.99±2.44	0.122±0.009	0.303±0.03
C1	7.14±0.88	10.08±0.59	0.024±0.005	0.006±0.002
N2	5.38±0.58 (P<0.0001)	2.82±0.31 (P<0.0001)	0.20±0.04 (P<0.222)	0.049±0.005 (P<0.0001)
U2	76.1±2.55 (P<0.0001)	93.89±1.78 (P<0.0001)	0.140±0.003 (P<0.0001)	1.163±0.08 (P<0.0001)
C2	8.67±0.67 (P<0.0004)	7.93±0.59 (P<0.0001)	0.035±0.007 (P<0.0008)	0.047±0.015 (P<0.0001)
N3	5.98±0.70 (P<0.0001)	2.90±0.15 (P<0.0001)	0.23±0.05 (P<0.0143)	0.038±0.004 (P<0.0001)
U3	98.62±6.08 (P<0.0001)	108.13±2.26 (P<0.0001)	0.187±0.005 (P<0.0001)	1.385±0.04 (P<0.0001)
C3	10.96±0.68 (P<0.0001)	8.06±0.76 (P<0.0001)	0.032±0.006 (P<0.0046)	0.038±0.014 (P<0.0001)
N4	6.93±0.49 (P<0.0001)	2.01±0.07 (P<0.0001)	0.44±0.07 (P<0.0001)	0.056±0.006 (P<0.0001)
U4	87.27±3.25 (P<0.0001)	99.42±4.59 (P<0.0001)	0.219±0.020 (P<0.0001)	1.605±0.07 (P<0.0001)
C4	10.51±0.63 (P<0.0001)	6.78±0.48 (P<0.0001)	0.095±0.008 (P<0.0001)	0.075±0.020 (P<0.0001)

4. Conclusion

In conclusion, it can be inferred that administration of garlic and mustard alone and in combination, to gastric cancer and gastric ulcer tissues effectively enhances the activities of GST and SOD enzymes and also could increase the level of important intracellular antioxidant, GSH in diseased condition, thereby, agreeing with previous reports that the protective effects of garlic and mustard extracts involves maintenance of antioxidant capacity in preventing the tissues against oxidative stress and thereby eliminating cancer risks. These spices could also help in reverting the effect of nitric oxide, which leads to cancer progression, through inhibition of NOS activity. Thus, this in vitro observation on human can be extrapolated to in vivo study, by administration of beneficial bioactive principles from natural plant based sources.

5. Acknowledgement

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