IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Comparative Analysis Of Oxidative Stress Parameters In *Zingiber Officinale* And *Piper Nigrum* In DMBA Induced Oral Carcinoma Rat Model And Evaluating Their Therapeutic Potential As Antiproliferative Agents.

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ABSTRACT: Cancer, a multifactorial disorder, arises due to defect in genetic makeup and exposure to unfavourable environmental conditions. Oral carcinogenesis proceeds through a spectrum of pathological lesions including hyperplasia, dysplasia, carcinoma in situ and invasive carcinoma. Phytochemicals have been fascinating scientists due to their property in altering cell cycle control and regulation, apoptosis, invasion, angiogenesis, interference with blood vascular system and metastasis. Phytochemicals are innovative alternatives and upcoming stratagem and alternatives with reduced and lessened toxic effects of chemical drugs. These agents can be topical and systemic although several of them have been underwrote for the studies of skin and mammary carcinogenesis, only few studies have been conducted in inhibition of oral carcinogenesis. Ginger is extensively employed in Chinese, Ayurvedic, Unani medicines and home remedies since antiquity for many ailments together with pain, inflammation, and gastrointestinal disorders. Our current study is intended to examine the status of natural antioxidant enzymes SOD, LPO and Catalase in DMBA administered rats as well as *Zingiber officinale* and *Piper nigrum* fed group and perform its comparative study.

KEYWORDS- Carcinogenesis, phytochemicals, *Zingiber officinale, Piper nigrum*, oxidative stress, catalase, lipid peroxidase.

INTRODUCTION

Oral cancer is the sixth most common cancer worldwide (Gil et al., 2008). Almost 90% of all oral cancers are categorized as squamous cell carcinoma (SCC) (Attar et al., 2012). In India, 20 per 100000 population are affected by oral cancer which accounts for about 30% of all types of cancer (Nair et al., 2005) Cancer, a multifactorial disorder, arises due to defect in genetic makeup and exposure to unfavourable environmental conditions. Phytochemicals have been fascinating scientists due to their property in altering cell cycle control and regulation, apoptosis, invasion, angiogenesis, interference with blood vascular system and metastasis. They have proved their efficacy in single treatment procedures or in connotation with other chemo preventive agents. Phytochemicals can be largely classified into vitamins, carotenoids and food polyphenols phenols like flavonoids, phytoalexins, sulphur surplus compounds and phenolic acid indoles. Phytochemicals are innovative

alternatives and upcoming stratagem and alternatives with reduced and lessened toxic effects of chemical drugs. These agents can be topical and systemic although several of them have been underwrote for the studies of skin and mammary carcinogenesis, only few studies have been conducted in inhibition of oral carcinogenesis. Primary diagnosis and prompt preventive strategies should therefore be contributed to advance the quality of life of patient. Despite the abundant literature on molecular mechanisms of phytochemicals very scarce and insufficient of them have been put down in scientific and clinical trials. We in these series of experiments are evaluating the therapeutic potential of both the screened herbs and comparative analysis of their oxidative stress markers enzymes.

Zingiber officinale Ginger (*Z. officinale*) rhizomes are regularly used in foods and beverages for their distinguishing pungency and piquant flavor. The bioactive constituents of ginger have been acknowledged. Many of the advantageous pharmacological effects of its ingredients have been experimentally verified in current years. Ginger compounds are potent antioxidants, and subsequently, ginger extracts execute promising anti-inflammatory and cancer precautionary effects. Both gingerols and shogaols display a host of biological activities, ranging from anticancer, anti-oxidant, antimicrobial, anti-inflammatory and anti-allergic to numerous central nervous system activities. potent carcinogen which on metabolism form diol epoxides and other reactive oxygen species (ROS). These toxic reactive oxygen species can induce chromosomal aberrations and upsurge number of micronuclei through oxidative base damage and breakdown of DNA strand, leads to mutagenesis and carcinogenesis (Satouchi etal., 2007). In this study, the dried ginger officinale and piper nigrum nigrum extracts were fed to dmba induced oral male wistar rats to evaluate the antigenotoxic, anti-cell proliferative, cytoprotective potential on 7, 12- Dimethyl Benz [a]anthracene (DMBA) induced genotoxicity and oral carcinoma in male wistar rats.

Piper Nigrum: Piper nigrum (PN) is well known for its cytotoxic and pharmacological profits. However, there is nominal documented evidence about its cytotoxic efficacy in contrast to various carcinoma model. Of all the plants explored over these long years, the one exhibiting incredible potential through its bio valuable phytochemicals being curtained of its herbal profile is *P. nigrum* (PN) (black pepper aka king of spices) which belongs to the family Piperaceae. Although promising outcomes were also revealed by Piper longum and Piper beetle, in many carcinoma models cited in literature but there is no robust evidence related to this herbal extract with corroboration to oral carcinoma model. PN is the most commonly used and highly reviewed spice in the world, especially in India. It is loaded with plentiful pharmacological activities as it is proved to behave like an antihypertensive, anti-asthmatic, antimicrobial, antioxidant, anticancer, anti-inflammatory, and immunomodulatory activities.

Spices have been extensively used as condiments for thousands of years because of their flavor, sensitivity and color. Several spices have been used as medicinal plants in folk medicine for the conduct of various diseases because they contain numerous bioactive compounds and possess lot of advantageous health effects. These phytochemicals having antioxidant properties are acknowledged to produce anti-genotoxic consequence by plummeting toxic free radicals. In a number of studies, stress has been laid down on use of certain dietary ingredients for prevention of numerous drug and chemical induced genotoxicity in dissimilar phytoconstituents like Aloe Vera, Turmeric, lipoic acid, curcumin, gingiber, piperine, carotenoids and vit-C and vit-E. With such a circumstantial this study has been carried out to appraise any anti-mutagenic activity, anti-cell proliferative and cytoprotective activity of *Z. officinale* and *P. nigrum* excerpts against 7, 12-Dimethylbenz [a] anthracene (DMBA) induced genotoxicity oral carcinoma rat model.

Wistar rat model are used as a perfect prototypical for oral cancer research due to its oral anatomy, and more importantly because of genomic similarity between rodents and homosapiens thereby presenting a strong corroboration with the studies and its outcome. DMBA, a powerful organ specific carcinogen, can persuade oral carcinoma in the buccal tissue of wistar rats, which exhibit biochemical, molecular and histopathological resemblances to human oral cancer. DMBA arbitrates carcinogenesis through chronic inflammation, numerous mutations and genetic adjustments and via excessive generation of reactive oxygen species occurring throughout metabolic activation of DMBA. DMBA induced carcinogenesis is therefore frequently used to study the biochemical and molecular apparatuses of oral carcinogenesis in rat model as well as to assessment

of the chemo precautionary efficiency of the natural products and its dynamic constituents (Murata et al., 2004; Manoharan et al., 2013).

AIM AND OBJECTIVE

Our study included oxidative stress and biochemical parameters studies in *Zingiber officinale* and *Piper nigrum* fed cancer induced wistar rat group, with following objectives- To find out and report risk assessment, clinical features and management of severe DMBA induced carcinogenicity. To observe the changes in routine biochemical parameters like lipid peroxidation rates, activity of catalase and superoxide dismutase enzymes in tissue sample (Buccal, kidney and liver) because these basic routine biomolecules play a very important role in the metabolism and during pathological condition their fluctuation from normal range reflect the severity of illness. Nonfunctional plasma enzyme AST, is very important enzymes present in plasma, which help in the diagnosis and prognosis of various tissue or membrane damage, it's serum value help in the assessment of tissue damage. To know the status of natural antioxidant enzymes SOD, LPO and Catalase in DMBA administered rats as well as *Z. officinale* and *P. nigrum* administered rats.

MATERIAL AND METHODOLOGY

Herbal extract *Zingiber officinale* and *piper nigrum* was purchased from Amsar Pvt. Ltd. Indore, M.P. Carcinogen DMBA analytical standard was purchased from Sigma Aldrich Co. Ltd. St. Louis, USA. DMSO, EDTA, hydrogen peroxide, Sulphuric acid, Diethyl triamine pentaacetic acid, sodium dodecyl sulphate, TBA and pyrogallol, were purchased from E Merks Ltd. Mumbai India. All other chemicals were of technical grade and purchased from Loba Chemie, Mumbai India.

Male Wistar rats *Rattus Norvegicus* are used in this study. A total of 60 colony bred Swiss albino rats Rattus Norvegicus are used in this study. Rats were raised under laboratory conditions from an initial cohort of 20 rats via inbreeding for a period of 8 months. The entire experiment was scheduled in triplicates. Rats of 8-10 weeks of age, with average body weight of 150-180 gm, were obtained from Institute of Animal health and Veterinary and Biological Products, Rasalpura, Mhow, Madhya Pradesh. The animals were maintained at 22±3 °C, with 50-70% relative humidity and 12:12 hrs of light and dark cycles and were kept in well-ventilated cages. The animals were fed with calculated amount of laboratory pellet diet procured from government agricultural college, Indore, India, and water ad libitum. Maintenance and cleaning were carried out twice a week and drinking water was replaced weekly or prior to that if required. The animals were observed daily to assess their general health. Animals were maintained as per the guidelines laid down by departmental ethical committee for handling and maintenance for experimental animals and the committee for the purpose of control and supervision on experiments in animals (CPCSEA NO-3565/IAEC/2014).

GROUP	DRUG	DOSE	MODE OF ADMISITRATION
CONTROL	Dimethyl sulfoxide (DMSO)	----------	Painting on oral epethelium
DMBA	7,12 Dimethylbenz[a]anthracene	0.2%	Painting on oral epithelium
T1	*Zingiber offficinale*	500mg/kg BW	Standard diet in air dried pellet supplemented with desired extract concentration
T2	*Piper nigrum*	100mg/kg BW	Standard diet in air dried pellet supplemented with desired extract concentration

TABLE: REPRESENTATION OF EXPERIMENTAL DESIGN WITH DOSE ADMINISTRATION

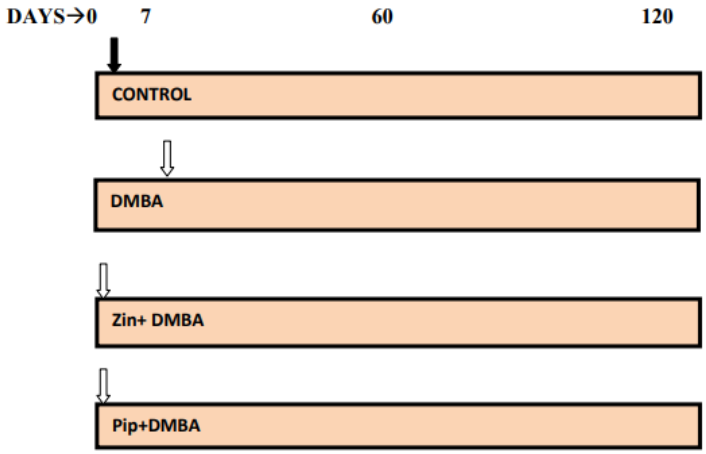


FIG 1: DOSE ADMINISTRATION SCHEDULE

On the day of termination (120th day), over-night fasted animals were sacrificed after exposing them to mild ether anaesthesia. Blood from each animal was collected and serum was isolated for estimation of various biochemical parameters. After exsanguinations, liver, Kidney, spleen and buccal tissue samples were quickly removed and washed in ice-cold phosphate buffer saline (PBS, 0.1 M, pH 7.4), weighed and homogenized in 4 volumes of 5mM phosphate buffer, pH 7.4 and centrifuged at 4000g for 25 minutes for estimation of SOD, LPO and CAT activities and protein contents.

AST (Aspartate Aminotransferase) or SGOT (Serum Glutamic-Oxaloacetate Transaminase) Activity. For the estimation of SGOT activity in serum samples, an enzymatic kit, an established protocol (Reitman and Frankel, 1957) was used. The coloured complex was read at 505 nm.

Catalase Activity was determined by method of Aebi (1991). The rate of decomposition of H2O2 catalase is measured spectrophotometrically at 405nm.

SOD activity was determined by the method of Marklund and Marklund (1974) modified by Nandi and Chatterji (1988). O.D. at 420 nm exactly after 1min 30sec and 3min 30sec and absorbance recorded per 2 minute.

Lipid Peroxidation in Tissue Lipid peroxidation was measured in tissue by method of Utley et al., (1967) or TBARS assay (Thiobarbituric acid reactive substances). Reading measured colorimetrically at 540nm (extinction coefficient, E= 1.56 X10^5), using a Shimandzu UV-1700 spectrophotometer

Protein Analysis was estimated in all samples by the method described by Lowry et al., (1951).

RESULT

Biochemical Parameters

Antioxidant Stress parameter in tissues (Buccal, Kidney and Liver)

Reactive oxygen species (ROS) are the by-products generated during the respiratory and metabolic reactions in our body. In cancer cases, the cells generate reactive molecules rapidly to accelerate their growth, so these molecules can act as a cancer marker. In study which we had performed there is involvement of both enzymatic and non-enzymatic antioxidant defence to cope up with oxygen free radicals. Theses parameters were SOD, CAT, LPO, and AST.

The status of antioxidants was significantly decreased in tumor bearing animals (Group 2) as compared to control animals. Oral administration (feed mixed) of Zingiber and Piper to DMBA painted animals (group T1 and T2 respectively) significantly brought back the concentration of antioxidants to normal status.

There is a general increase in the LPO levels of liver, kidney and buccal tissues exposed to DMBA intoxication as observed in DMBA group (group 2). The LPO values in liver, kidney and buccal tissue in DMBA

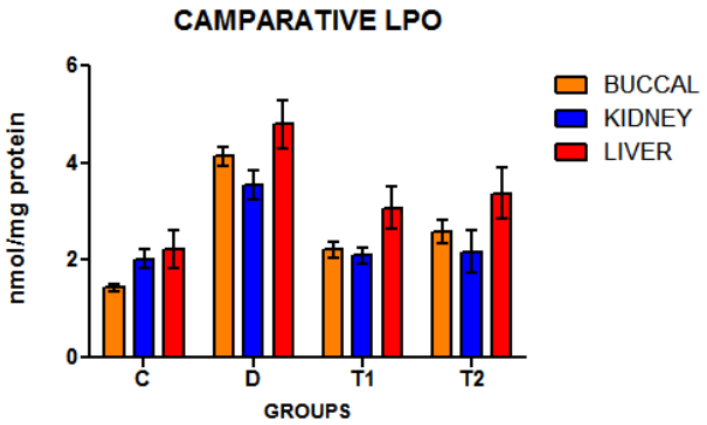
administered group was found to be 4.793±0.497, 3.547±0.307, and 4.135±0.193. The LPO values in case of Liver, kidney and buccal tissues in group T1 and T2 which were administered with Z. officinale and P. nigrum as well as intoxicated with DMBA were comparable to the control values.

The LPO values in case of liver, kidney and buccal tissue in group T1 was found to be 3.080±0.427, 2.095±0.167 and 2.213±0.175 which are comparable and consistent to the LPO values 2.230±0.387, 2.030±0.187 and 1.433±0.084. The LPO values in case of liver, kidney and buccal tissues in group T2 was found to be 3.378±0.527, 2.172±0.437 and 2.592±0.236 which are comparable to the LPO values of control group with the values 2.230±0.387, 2.030±0.187 and 1.433±0.084.

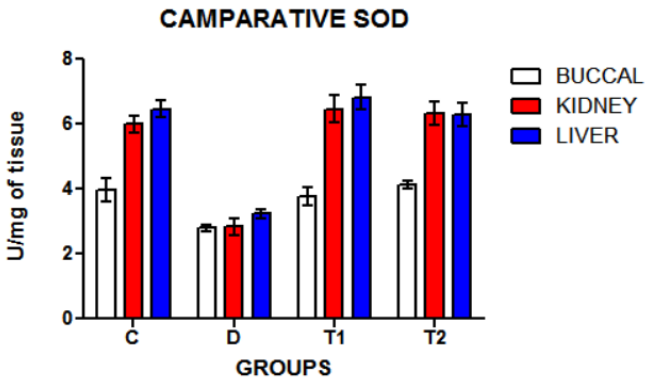
Administration of *Zingiber officinale* (500mg/kg BW) and *Piper nigrum* (100mg/kg BW) in the T1 and T2 group caused significant reduction of LPO values as compared to the DMBA intoxicated group. The significant decreases in LPO values were found to be consistent and comparable to the LPO values of the control group.

Administration of *Z. officinale* (500mg/kg BW) and *P. nigrum* (100mg/kg BW) manifested modulatory mechanism and reverted back the levels of both enzymes i.e., superoxide dismutase and catalase near to normal in the liver, buccal tissue and kidney of animals in group T1 and T2 and is found to be comparable to control group. In the present study, elevated phase II enzyme levels in skin and liver of experimental animals were recorded after *Z. officinale* and *P. nigrum* administration.

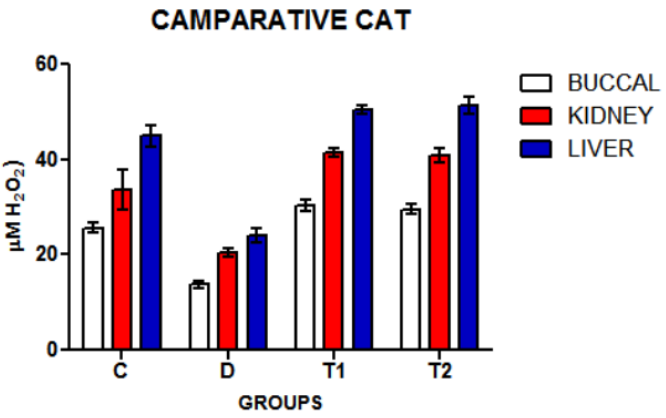
SOD accelerates the dismutation of superoxide anions to hydrogen peroxide and finally CAT, Carcinogen treatment reduced the levels of SOD by generating ROS, whereas the free radical scavenging action of *Z. officinale* and *P. nigrum* restored the same toward normal in the experimental animals. The LPO values in case of the status of antioxidants in the buccal mucosa of the control and experimental animals in each group revealed acute disturbance in antioxidant status in case of experimental animals (SOD and CAT were decreased while LPO was increased). Male wistar rats treated with *Zingiber* and *Piper* shows no significant difference in antioxidant status as compared to control group. Oral administration of *Zingiber* (500mg/kg BW) and *Piper* (100mg/kg BW) brought back the activities of detoxification agents to normal status. There is significant enhancement in the levels of SOD and Catalase in the T1 and T2 group. The increase in enzyme activity in group T1 and group T2 effectively reduced the generation of ROS and LPO in the skin and thus might reduce the incidences tumor formation on the painted areas. SOD and Catalase are acting as mutually supportive antioxidative enzymes which provide protective defense against reactive oxygen species.



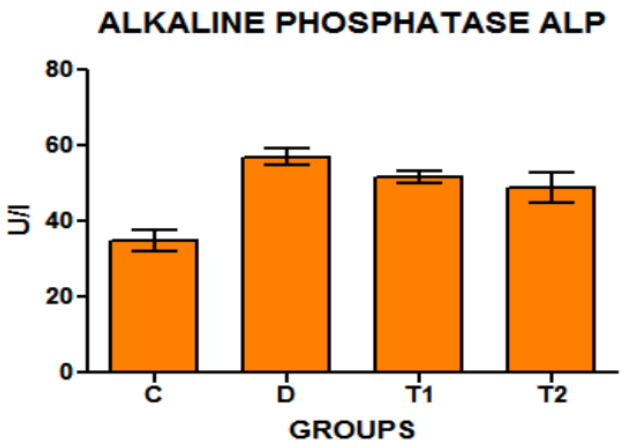
Graph 1: Comparative changes in concentration of liver, buccal and kidney tissue LPO (nmol/mg protein) in experimental animals following carcinogen and herbal extract administration in male rats. Each vertical bar represents mean ± S.E.M (n=5), as compared to the control values.



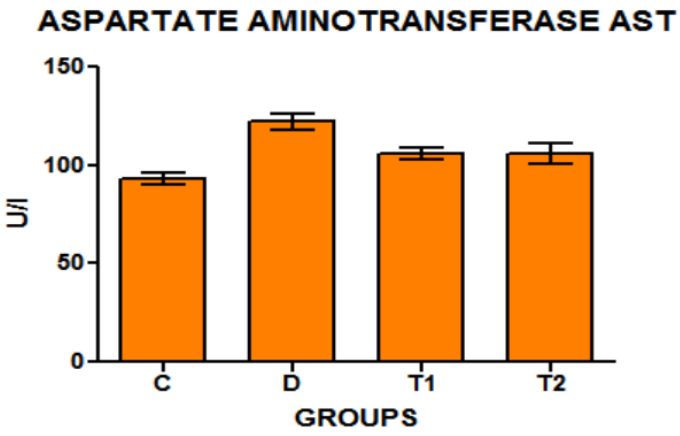
Comparative analysis of superoxide dismutase alteration in all experimental groups. Each vertical bar represents mean±S.E.M (n=5), as compared to the control values.



Comparative changes in concentration of liver, buccal and kidney tissue CAT (µm H2O2) in experimental animals following carcinogen and herbal extract administration in male rats. Each vertical bar represents mean±S.E.M (n=5), as compared to the control values



Changes in concentration of serum ALP (nmol/mg protein) in experimental animals following carcinogen and herbal extract administration in male rats. Each vertical bar represents mean±S.E.M (n=5), as compared to the control values.



Changes in concentration of serum AST (nmol/mg protein) in experimental animals following carcinogen and herbal extract administration in male rats. Each vertical bar represents mean±S.E.M (n=5), as compared to the control values.

DISCUSSION:

During the carcinogenic process lipid peroxidation is increased and complex reactive compounds (MDA) are obtained. These products are highly mutagenic and carcinogenic. The agents that reduce the production of free radicals are chemoprotective. LPO is produced during life processes in a chain reaction initiated by free radicals produced. Malondialdehyde is the lipid electrophile generated from peroxidation, and an increase in its levels is an alarming sign of various diseases, including cancer. Topical application of DMBA to animals increases the production of ROS and ultimately leads to higher LPO levels in and liver, as observed in the present study. In contrast, Zingiber and Piper rich in gingerols, shogaols, piperine and piperidine protect the cellular membranes, and its other phytochemicals scavenge free radicals and inhibited LPO level in experimental animals.

The dose of *Z. officinale* and *P. nigrum* did not alter the final body weight and the weights were comparable to that of control. Although percentage differences in mean terminal body weights between the dietary control group and the group supplemented with DMBA were different. A significant protection against oral carcinogenesisis conferred by *Z. officinale* and *P. nigrum*. At study termination, oral cancer incidence in rats receiving chronic dietary exposure to *Z. officinale* and *P. nigrum* were essentially identical as compared to dietary control group. Finally, clinical studies (in-vivo) examining both the safety and efficacy of herbal extracts and their derived phytochemicals has been investigated and there is marked change in most of the clinical parameters (no reduction in weight) in treatment group. Oral administration of *Z. officinale* and *P. nigrum* (500 mg/kg body weight and 100mg/kg body weight) to DMBA-treated rats for 16 weeks inhibited the tumor incidence not in an absolute but qualified manner and restored the physical parameters near to normal levels. These findings were in orderliness with Rajendran et al., (2019). DMBA induced rats showed a significant reduction in body weight due to their cancer cachexia which is directly correlates to cancer progression in experimental subject perhaps this may be due to the damage of skeletal muscle and adipose tissue. *Z. officinale* and *P. nigrum* treatment opposes the loss of body weight by its counteractive and antioxidants property which is evident from the gross monitoring of weight from time to time during the entire schedule of 120 days.

Biochemical parameters. Antioxidant stress parameters in tissues (Buccal, Kidney and Liver). Our body is equipped with complex antioxidant system to relieve oxidative stress, but it is clear from our biochemical estimations that how reactive oxygen species, derived from oxygen and nitrogen lead to oxidative damage to tissues, which is clearly visible by doing histopathology. Oxidative stress is a conjoint mechanism and contributes in liver injury. An increased free radical generation and/or reduced antioxidants may lead to several biochemical and pathological complications which has been suggested to play key role in initiation of carcinogenesis. In this study, we investigated the chemo preventive effect *of Z. officinale* and *P. nigrum* on DMBA-induced carcinogenicity in rats. Oxidative metabolism of DMBA implicated the ROS production that capable of generating free radicals and depletion of antioxidants leads to peroxidation of membrane lipids results degeneration and/or tissue injury has been a sign of carcinogenesis. Data also indicated that an increased lipid peroxidation have been detected in oral carcinoma rat model. Concurrently, in our results *Z. officinale* and *P. nigrum* pre-treatment and post treatment promoted better results because of intoxication of carcinogen to rats. In early stages the herbal preparation of our interest would have actively combated whereas in post-treatment condition cancer can be in aggressive proliferation and malignant transformation state which allowed minimal therapeutic effects. Antioxidants are substances that detain, prevent or remove oxidative damage by chelating trace elements or by inhibiting the enzymes involved in free radical production. This goes in accordance with the findings of Hanasaki et al., which implies that endogenous antioxidants certainly combat the reactive free radicals. Our results are very well coinciding with earlier findings suggesting that reduced oxidative stress might be the reason of excessive utilization of antioxidants. Therefore, we could suggest that *Z. officinale* and *P. nigrum* has potent antioxidants and prooxidant properties which had positively attenuated the oxidative stress. In this study, *Z. officinale* and *P. nigrum* treatment actively counterbalances the toxic effects of ROS through inhibiting oxidative damage of cell membranes thereby maintaining the Catalase and SOD values of group T1 and T2 comparable to control groups and these findings very well correlated with Haque et al., 2018 and hence, the exact and complete antiperoxidative and lipid lowering mechanism could have resulted in inhibition of oral carcinogenesis. The LPO values in case of Liver, kidney and buccal tissues in group T1 and T2 which were administered with *Z. officinale* and *P. nigrum* as well as intoxicated with DMBA were comparable to the control values. These investigations in case of oxidative stress markers LPO were in accordance to Kavitha et al., 2019 but were not consistent and different with Dogan et al., 2019.

Plasma Marker Indices: Alkaline Phosphatase (ALP) ALP is a ubiquitous enzyme and is mainly confined in liver serum ALP activity is potentially a very useful indicator for detection of malignancies but its status in case of oral squamous cell carcinoma is less explored. In our studies as we have explored and confirmed an increase in ALP values in carcinoma rat group as compared to control group suggesting that ALP has been proven to be a prognostic factor in oral carcinoma. This study did not provide direct evidence for either possibility, but indirect evidences favouring production of ALP by tumor cells had been evaluated. These investigations are in orderliness and validates with Kim et al., 2017. As in group T1 and T2 treated with *Z. officinale* and *P. nigrum* leads to an increase in ALP level, but is comparable and very well co-relates to the

control group. The possible mechanism lies with the fact that these herbal extracts subsided the proliferation rate and proved to be antiproliferative thus minimizing liver stress and thus reduction in ALP levels as compared to carcinoma group. In case of groups T1 and T2 there is significant elevation in serum ALP values as compared to the control group. As compared to the control group there is slight increase in the serum ALP levels in T1 and T2. Aspartate Transaminase (AST) AST catalyses the reversible transfer of an α-amino group between aspartate and glutamate and, as such, is an important enzyme in amino acid metabolism. In case of groups T1 and T2 there is significant elevation in serum AST values as compared to the control group, while in case of carcinoma group there is high fold increase in AST level, exploring the dimension of high proliferation rate of cancer cells. As in cancer there is complete unscheduling of cells in terms of cell cycle regulation and the nucleotides required are at a very high level. The precursor molecules would be generated from amino acid catabolism in which primary catabolic enzymes like AST would be involved at a very high level leading to very high level of AST in cells undergoing uncontrolled cell division as opposed to normal cell population having optimal value of AST. These findings very well corroborated with Mansingh et al., 2019, but were inconsistent with Rajasekaran et al., 2015.

Chemotherapy is a widely employed anticancer therapy. However, the beneficial potential of chemotherapy antagonist to cancer is seriously dissatisfactory due to nonspecific drug distribution and heterogeneity of cancer. In the studies we performed an approach for improvised combinatorial therapy which could be combed, in future and adding one more expectant dimension that is clubbing with nanoparticle drug delivery system which will certainly offers bull's eye specific action, a vital parameter for cancer drug delivery system.

A WAY TO COMBINATORIAL THERAPY: A therapy based on chemotherapy in combination with our extracts of interest (*Z. officinale* & *P. nigrum*) can certainly reduce the pain and side effects, where optimization of drug is a decisive factor. Phytochemicals can be commingled with chemo preventive agents with superior clinical applications in preventing oral cancer. They may also facilitate us to discover new preventive strategies for cancer management and aid in improvising patients continued existence.

PHYTOCHEMICALS AS ANTIPROLIFERATIVE AGENTS: The current research work highlighted the potential and diversity of phytochemicals present in *Z. officinale* & *P. nigrum* (shogaols, piperidine, gingerols, gingerones) as antiproliferative agents. The promise of these phytochemicals acting as anticancer agent is evaluated on the basis of its ability to target the cells undergoing uncontrolled proliferation by their preferential inhibition of the proliferation of cancer cells over normal ones.

EXTRACTS AS ROS QUENCHER: Antioxidant quotient of extracts of interest certainly counterbalances the oxidative microenvironment. *Z. officinale* & *P. nigrum* quenched the reactive oxygen species (ROS) activity. Therefore, antioxidant therapies may avert the adverse effects of chemotherapy. They prevent cellular damage of normal organs and tissues by reacting with oxidizing free radicals. In the present research the screened herbal extracts are found to have potential cytoprotective and antiproliferative activity and unquestionably is a promising treatment strategy and substitute for cancer research, but some more aspects will be playing decisive role like mode of administration, dose optimization, stage of tumour, antioxidant factor of herbal extracts, active phytochemicals, mode of action with precision on target tissues and many more which we have already worked on and validated as well. This entire work is of great practical significance in public health sector if we look at the statistical figures of increasing carcinoma cases over the past decade.

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