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CHEMOPREVENTIVE EFFICACY OF *ADHATODA VASICA* LEAVES IN 7,12-DIMETHYLBENZ[A]ANTHRACENE INDUCED ORAL CARCINOGENESIS

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ABSTRACT

Imbalance in oxidant and antioxidant status is one of the commonly observed phenomenon in several pathological conditions including cancer. Also, defect in detoxification cascade leads to accumulation of toxic metabolites, which cause mutation in DNA and leads to neoplastic transformation. Aim of the present study is to focus the chemopreventive potential of *Adhatoda vasica* leaves during DMBA induced hamster buccal pouch carcinogenesis by analyzing the status of lipid peroxidation by products (TBARS), antioxidants (Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and Vitamin E) and detoxification agents (cytochrome p450, cytochrome b5, glutathione-s-transferase (GST), reduced glutathione (GSH), glutathione reductase (GR), DT-diaphorase and oxidized glutathione (GSSG)). Topical application of DMBA to the buccal pouches of hamsters, thrice a week for 14 weeks, developed well differentiated squamous cell carcinoma. At the end of the experimental period, we noticed an imbalance in oxidant and antioxidant status as well as defect in the detoxification mechanism in hamsters treated with DMBA alone. Though oral administration of ethanolic extract of *Adhatoda vasica* leaves (AVELet) to hamsters treated with DMBA, suppressed the tumor formation, we noticed hyperplasia and dysplasia. AVELet also modulated the status of lipid peroxidation, antioxidants and detoxification cascade in favour of delaying the tumor formation in hamsters treated with DMBA. The present study thus highlights the tumor preventive potential of AVELet, which could be attributed to its antioxidant potential during DMBA induced oral carcinogenesis. Further studies are in progress to isolate and characterize the bioactive principles from the leaves of *Adhatoda vasica*.

KEYWORDS: *Adhatoda vasica*, Chemoprevention, Antioxidants, Detoxification agents, DMBA, Oral cancer.

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INTRODUCTION

Oral cancer, a major life threatening disfiguring disease, affects around 3, 00, 000 people every year worldwide and has a low survival rate and poor life quality. Oral cancer constitutes around 30% of all cancers in developing countries including India. Tobacco and alcohol consumption attributes mainly to the increasing incidence of oral cancer worldwide. Early diagnosis of oral cancer would improve the 5 year survival outcome of the patients. 7,12-dimethylbenz[a]anthracene (DMBA), a potent carcinogen, induces neoplastic transformation by causing oxidative stress, DNA damage and abnormal biological events in the cells. DMBA is widely preferred in several experimental studies to investigate the morphological, biochemical, physiological and molecular alterations occurring in the cells. Oxidative stress arises due to imbalance in oxidant and antioxidant status and has been implicated in the deterioration of normal cellular processes that have a role in cell differentiation and proliferation. Lipid peroxidation, a process that occurs due to oxidative deterioration of lipids, has been implicated in the pathogenesis of carcinogenesis. Multicellular organism has, however, inbuilt with enzymatic and non-enzymatic antioxidant defense mechanism to counteract oxidative stress. Antioxidants protect oxidative tissue damage by scavenging excessively generated ROS in the system. SOD, CAT and GPx, the enzymatic antioxidants and Vitamin E, Vitamin C and glutathione, the non-enzymatic antioxidants, play a pivotal role against ROS mediated oxidative damage. The liver has a detoxification cascade to actively metabolize and excrete the toxic metabolites of the xenobiotic agents. While phase I enzymes contribute their role in the metabolic activation of the procarcinogens, phase II enzymes participate in the excretion of toxic metabolites in conjugation with reduced glutathione. Any deviation in the balance between these detoxification cascades could result in neoplastic transformation. India is gifted with a large number of medicinal plants, which are used in traditional medicine for the treatment of several chronic diseases including cardiovascular diseases, obesity, diabetes and cancer. Experimental and clinical studies focused the therapeutic potential of several phytochemicals against cancer. Medicinal plants are a rich source of antioxidants, flavanoids, phenolic compounds and terpenoids. *Adhatoda vasica* is one such well known plant possess diverse pharmacological and biological activities including antioxidant, antimicrobial, antitussive, hepatoprotective and anticancer potential. Phytochemical analysis revealed the presence of alkaloids, quinazoline, vasicene, vasicinone, deoxyvasicine and phenolic compounds. Due to the presence of the above said phytochemicals in *Adhatoda vasica*, we made an attempt to explore the chemopreventive potential of *Adhatoda vasica* in DMBA induced hamster buccal pouch carcinogenesis. The present study was designed to assess the chemopreventive potential of *Adhatoda vasica* by examining histopathological abnormalities as well as analyzing the enzymatic and non-enzymatic antioxidants, and detoxification agents in hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS

(i) Plant extract preparation

Before carrying out the experimental studies, the *Adhatoda vasica* leaves were first submitted to Botany department, Annamalai University to get and confirm taxonomic authentication. The ethanolic extract was then prepared according to the method of Hossain et al. A semisolid extract (7%) obtained was suspended in distilled water and orally fed to the experimental animals at a dose of 100mg/kg bw.

(ii) Experimental design

The Annamalai University ethical clearance committee [Reg.no:160/1999/CPCSEA] for animal experimental studies approved the experimental design. The animals were categorized as vehicle treated control hamsters, hamsters treated with DMBA alone, DMBA + AVELet treated hamsters and hamsters treated with AVELet alone. Each group contained 10 hamsters and the experimental protocol was designed as...
follows. Vehicle treated control: Topical application of liquid paraffin alone three times a week for 14 weeks in the left buccal pouches of hamsters. Hamsters treated with DMBA: Topical application of DMBA (0.5% DMBA in liquid paraffin) three times a week for 14 weeks in the left buccal pouches of hamsters. DMBA + AVELet treated hamsters: In addition to DMBA treatment as mentioned above, hamsters received oral administration of AVELet (100 mg/ kg bw three times a week for 14 weeks) on alternate days of DMBA painting. AVELet alone treated hamsters: Oral administration of AVELet alone (100 mg/ kg bw) three times a week for 14 weeks. The animals were sacrificed by cervical dislocation and the biochemical analysis was done in plasma, liver and buccal mucosa of the experimental hamsters. Histopathological studies were carried out to confirm the precancerous and cancerous lesions in the oral cavity of experimental hamsters.

(iii) Biochemical estimations
Lipid peroxidation by products, thiobarbituric acid reactive substances (TBARS), were assayed by the method of Yagi \(^{22}\) and Ohkawa \(^{23}\) in the plasma and tissues respectively. The antioxidant status was measured according to the methods of Kakker et al \(^{24}\) (SOD), Sinha \(^{25}\) (CAT), Rostruck et al \(^{26}\) (GPx) and Desai \(^{27}\) (Vitamin E). The status of detoxification agents in the liver and buccal mucosa were estimated according to the method of Omura and Sato \(^{28}\) (cytochrome p\(^{450}\) and cytochrome b\(_5\)), Habig et al \(^{29}\) (GST), Beutler and Kelley \(^{30}\) (GSH), Carlberg and Mannervik \(^{31}\) (GR), Ernster \(^{32}\) (DT-diaphorase) and Tietze \(^{33}\) (GSSG).

RESULTS
The macroscopic examination of buccal mucosa revealed 100 % tumor formation with an increase in tumor burden (1507.50 mm\(^3\)) and tumor volume (418.75 mm\(^3\)) in hamsters treated with DMBA alone as compared to control hamsters (Table 1). Histopathological analysis confirmed the tumors as well differentiated squamous cell carcinoma (Fig 1b). Hamsters treated with DMBA + AVELet exhibited mild to moderate precancerous lesions such as hyperplasia, dysplasia and hyperkeratosis (Fig 1c). We have however noticed no tumor formation during the experimental period in DMBA + AVELet treated hamsters.

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<tr>
<td>Tumor burden (mm(^3)) /animals</td>
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Tumor volume was measured using the formula, \(v = \frac{4}{3} [D1/2] [D2/2] [D3/2]\) where D1, D2 and D3 are the three diameters (mm\(^3\)) of the tumor. Tumor burden was calculated by multiplying the tumor volume and the number of tumors / animals.
Figure 1

**Histopathological changes in the buccal mucosa of control and experimental hamsters in each group (Magnification 20 X)**

(a & d): Photomicrographs showing well-defined intact epithelium from control and AVELet alone treated hamsters respectively (H & E, 20X). (b): Photomicrographs showing well-differentiated squamous cell carcinoma with keratin pearls in hamsters treated with DMBA alone (H & E, 20X). (c): Photomicrographs showing mild to moderate precancerous lesions such as hyperplasia, dysplasia and hyperkeratosis in hamsters treated with DMBA+ AVELet (H & E, 20X).

While phase I and phase II detoxification agents are significantly increased except GSSG in the buccal mucosa of hamsters treated with DMBA (Fig 2), we noticed an increase in phase I and decrease in phase II detoxification agents in the liver of hamsters treated with DMBA (Fig 3). DMBA + AVELet treated hamsters showed a near normal range of the above mentioned biochemical variables in both the liver and buccal mucosa.

Figure 2

**Status of phase I and phase II enzymes in the buccal mucosa of control and experimental hamsters in each group.**

Values are expressed as mean ± standard deviation (S.D) for ten hamsters in each group. Values that do not share a common superscript between the groups differ significantly at p< 0.05 (DMRT). X- micromoles of cytochrome p450; Y- micromoles of cytochrome b5; A – micromoles of 1- chloro 2,4 dinitrobenzene (CDNB) / reduced glutathione conjugate formed per minute.
Figure 3
Status of Phase I and Phase II detoxification agents in the liver of control and experimental hamsters in each group

Values are expressed as mean ± standard deviation (S.D) for ten hamsters in each group. Values that do not share a common superscript between the groups differ significantly at p < 0.05 (DMRT). X- micromoles of cytochrome p450; Y- micromoles of cytochrome b5; A – micromoles of 1-chloro 2,4 dinitrobenzene (CDNB) / reduced glutathione conjugate formed per minute.

While lipid peroxidation by products (TBARS) was increased in plasma (Fig 4), the status was found to be decreased in the buccal mucosa of hamsters treated with DMBA alone (Fig 5). Furthermore, the antioxidant status was also revealed different strategies. While both enzymatic and non-enzymatic antioxidants were decreased in the plasma (Fig 6), we noticed increased levels of Vitamin E and GPx activity and decreased activities of SOD and CAT in the buccal mucosa of hamsters treated with DMBA alone (Fig. 7)
Figure 4

Status of TBARS in the plasma of control and experimental hamsters in each group

Values are expressed as mean ± standard deviation (S.D) for ten hamsters in each group. Values that do not share a common superscript between the groups differ significantly at p < 0.05 (DMRT)

Figure 5

Status of TBARS in the buccal mucosa of control and experimental hamsters in each group

Values are expressed as mean ± standard deviation (S.D) for ten hamsters in each group. Values that do not share a common superscript between the groups differ significantly at p < 0.05 (DMRT).
**Figure 6**
Status of plasma antioxidants in control and experimental hamsters in each group

Values are expressed as mean ± standard deviation (SD) for ten hamsters in each group. Values that do not share a common superscript between the groups differ significantly at p< 0.05 (DMRT), A – amount of enzyme required to inhibit 50% NBT reduction; B – micromoles of hydrogen peroxide utilized/s; C – micromoles of glutathione utilized/min.

**Figure 7**
Status of buccal mucosa antioxidants in control and experimental hamsters in each group

Values are expressed as mean ± standard deviation (SD) for ten hamsters in each group. Values that do not share a common superscript between the groups differ significantly at p< 0.05 (DMRT), A – amount of enzyme required to inhibit 50% NBT reduction; B – micromoles of hydrogen peroxide utilized/s; C – micromoles of glutathione utilized/min.
DISCUSSION

Chemoprevention, an appealing experimental strategy, deals with the anticancer efficacy of natural products or synthetic entities. Extensive chemoprevention studies on various experimental cancer models clearly highlighted that chemopreventive agents explored their anti-cancer efficacy through antioxidant, anti-inflammatory, anti-cell proliferative and apoptotic potential as well as through their modulating effect on detoxification cascade in favour of the suppression or inhibition of tumorigenesis. In the present study, we have assessed the chemopreventive potential of *Adhatoda vasica* leaves in DMBA induced oral carcinogenesis by utilizing the status of lipid peroxidation, antioxidants and detoxification agents as biochemical end points. Macroscopic as well as microscopic examinations revealed a promising anticancer potential of *Adhatoda vasica* during DMBA induced oral carcinogenesis. Histopathological results explored that *Adhatoda vasica* leaves has the potential to delay the formation of oral tumors as evidenced by moderate dysplasia and hyperplasia in the buccal mucosa of DMBA + AVELet treated hamsters. The results also consistent with the present biochemical findings. Cytochrome P and b5 effectively exert their role in the metabolic activation of procarcinogens into their ultimate carcinogenic metabolites. GST, GR and GSH are involved in the excretion of active toxic metabolites that formed during phase I reactions. The status of phase I and phase II detoxification agents in the liver of DMBA + AVELet treated hamsters suggest that *Adhatoda vasica* significantly improved the detoxification mechanism towards the protection of tissue damage by excreting the active metabolite of DMBA. Thus, *Adhatoda vasica* has the ability to rectify the imbalance in the status of liver phase I and phase II detoxification agents in DMBA treated hamsters. We, however, noticed controversial findings in the buccal mucosa, where both phase I and phase II detoxification agents were increased in hamsters treated with DMBA alone. This might be due to frequent topical applications of DMBA (three times a week for 14 weeks) in the buccal mucosa, which enhanced the activities of phase I agents to metabolically active DMBA with subsequent compensatory enhancement of phase II agents to detoxify the carcinogenic metabolites of DMBA. *Adhatoda vasica* leaves rectified this defect also in hamsters treated with DMBA. Reactive oxygen species play a dual role depending on its physiological concentration. While they have a protective role at normal physiological concentrations, they have a destructive role at higher concentrations. Excessive generation of ROS in the biological systems mediates oxidative DNA damage, which result in neoplastic transformation. Biological system, however, has a well defined antioxidant mechanism to combat the harmful effects of ROS. However, under pathological conditions such as cancer, antioxidants are defenseless and ROS has thus upper hand. Excessive generation of ROS, as evidenced by increased plasma TBARS, accompanied by decreased levels of non-enzymatic and enzymatic antioxidants confirmed the imbalance in oxidant and antioxidant status in hamsters treated with DMBA alone. Oral administration of AVELet at a dose of 100 mg/kg bw to DMBA treated hamsters rectified this defect in the balance and improved the defense mechanism. Another interesting observation in the present study is the observation of lower lipid peroxidation by products, TBARS, in tumor cells accompanied by a decrease in SOD and CAT activities and increase in GPx activity, GSH and vitamin E content. This is probably due to high rate of cell proliferation as compared to their adjacent normal cellular counterpart. The inverse association between the rate of cell proliferation and lipid peroxidation has been well documented. Furthermore, low availability of PUFA, the substrate of lipid peroxidation, in tumor cells has also been focused as a reason for low TBARS content in tumor tissues. At the same time, increased generation of H2O2 and OH radicals in tumor cells was documented as causative factors of decreased activities of SOD and CAT. GSH and GPx play crucial role in the regulation of cell proliferation in addition to their role as antioxidants. Increased activity of GPx accompanied by an increase in GSH content has been documented in cancerous conditions.
Also, the tumors sequester essential nutrients, including vitamin E for their rapid growth from the circulation. Our results also lend credibility to these previous findings. Hamsters treated with DMBA + AVELet showed a near normal pattern of lipid peroxidation and antioxidants in the buccal mucosa, which suggest that Adhatoda vasica has the potential to rectify the defect in the oxidant-antioxidant status during DMBA induced oral carinogenesis.

CONCLUSION

The present study thus highlights the antioxidant potential as well as modulating effect of Adhatoda vasica leaves on detoxification agents in DMBA induced hamster buccal pouch carcinogenesis. The antioxidant potential of Adhatoda vasica leaves is probably due to the presence of antioxidant components such as alkaloids, quinazoline, vasicine, vasicinone, deoxyvasicine and phenolic compounds. Further effort has therefore been taken to investigate and isolate the active components from Adhatoda vasica leaves that are responsible for anticancer and antioxidant activities.

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