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Study to Analyse the Chemotherapeutic Effect of Propolis And Withaferin-A on Benz (A) Pyrene Induced Lipid Peroxidation and Antioxidant System in Wistar Rats

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Female breast cancer has become the first and most common malignancy surpassing lung cancer, and the global incidence is reported to be high. In general, developing countries like India reports more cancer cases which have become a significant health burden. The currently available anticancer agents pose significant toxicities besides the development of resistance. Therefore, natural compounds with promising anticancer activity may be investigated. In the present study, we evaluated the combinational effect of propolis and withaferin A in female Wistar rats subjected to Benz(a)pyrene-induced breast cancer. Five groups of rats, each consisting of six animals, were used in the study. Group I (normal control), group II (cancer control) were treated with saline and benz (a) pyrene, respectively. Whereas group III, group IV and group V were intended to receive withaferin A, propolis individually and in combination. Finally, antioxidant levels of all groups were estimated in blood using spectrophotometrically. Our results revealed that the combined treatment with withaferin A and propolis was effective compared to their individual effect. This observation was supported by decreased lipid peroxidation. Additionally, the levels of both enzymatic and non-enzymatic were elevated compared to the rats in the groups that received individual treatment. Propolis and withaferin A combination effectively prevent the Benz (a) pyrene-induced mammary carcinogenesis. The underlying mechanism could be their synergistic antioxidant property.

Keywords: Benz (a) Pyrene; Breast cancer; enzymatic; Non-enzymatic; Propolis; Withaferin-A.

Breast cancer is the most common & most frequent type of cancer in the world among women. A recent analysis on GLOBOCAN 2020 estimates that 2.3 million new instances of cancer would be detected worldwide in 2020, making up 11.7% of all new cancer cases[1]. The breast cancer rate is low, and the mortality rate is higher in Asia.[2] Even though the increased rate is found in developed countries, the awareness, screening practices and diagnoses are relatively low in developing countries.[3] Due to rapid urbanization, population growth & ageing factors in Indian women, the incidence and mortality rate in India is increasing.[4] The free radicals mainly contribute to the development of pathogenesis in cancerous and non-cancerous diseases due to excessive exposure to toxins, leading to oxidative stress.[5] Bloodstream-related antioxidant status and lipid peroxidation

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help to estimate oxidative stress in cancer patients.[6] Caffeic acid phenethyl ester (CAPE), a principal constituent of propolis, exhibits anti-tumour, anti-inflammatory, and antioxidant properties.[7-8] *Withania somnifera*, a herb with bioactive compounds like withaferin-A (WA), has been successfully used in the Ayurveda system of medicine.[9] Its applications like anticoagulant, antipyretic, antioxidant, and analgesic are well known.[10-12] Also, WA has shown antiproliferative, apoptosis, mitochondrial membrane depolarization, caspase activation, migration inhibition and G2/M cell cycle arrest in various cancerous cells. Inhibiting MMP-2 and MMP-9 also causes antioxidant gene expression and activation of MAPK (mitogen-activated protein kinase)[12]. The lipid peroxidation and antioxidant inducing property of withaferin A and propolis combination were investigated in Wistar rats pretreated with benz (a) pyrene (BaP).

MATERIALS AND METHODS

Central Research Laboratory of Meenakshi Medical College & Research Institute, Enathur, Kanchipuram, was the study centre to carry out the experiments with Wistar rats. The study was conducted from October 2019 to June 2021. Institutional Animal Ethical Clearance was obtained on 03/07/2019 (IAEC No: 003/2019). Female Wistar rats of 150-200 gms were procured from the National Institute of Nutrition, Hyderabad, India. On alternate 12-hour light/dark cycles, they were kept in a controlled environment with temperature and humidity. All of the rats were provided with a regular pelleted food (Gold Mohr rat feed, Ms. Hindustan Lever Ltd., Mumbai) and unlimited access to water.

Benz (a) Pyrene,Withaferin-A and Propolis were obtained from Aldrich Sigma Chemical, Mumbai and the remaining chemicals from SRL Chemicals in Chennai. Enzymatic antioxidants like Superoxide Dismutase (SOD) were estimated as per the method described by Marklund and Marklund (1974)][23], Catalase (CAT) by Sinha (1972),[24]. Glutathione peroxidase (GPx) by Rotruck *et al.* (1973) method[25]. The non-enzymic antioxidants like reduced glutathione (GSH) were measured by using Moron *et al.* (1979) method[26]. Vitamin C was estimated by Omaye *et al.* (1979) method[27] and Vitamin E was performed by Desai (1984) method[28].

Experimental Procedure

Five groups of six animals each were created from the total number of animals.

Group I**:** Non-diseased animal group fed with saline

Group II **:** Disease control group:- Animals treated with benzo(a) pyrene (20 mg diluted in 0.5 ml of sunflower oil and 0.5 ml of saline in two mammary pads using "air pouch method") were given the drug twice a week for three months.

Group III**:** Orally Withaferin A (30mg/kg b.wt,) was administered to the Breast cancer bearing animals weekly once for four weeks.

Group IV **:** Ethanolic extract of propolis was orally (50 mg/kg body weight) fed to the breast cancer bearing animals for 30 days.

Group V **:** Both Withaferin A and ethanolic extract of propolis (as above) was administered to the breast cancer bearing animals.

Ethanolic extract of Propolis preparation

Propolis was extracted in a hermetically sealed glass jar using 95 percent v/v ethanol for 4 days at 37°C with periodic shaking. After filtering using Whatman Filter Paper #4, the ethanolic extract was heated to 60°C under decreased pressure before being evaporated in a rotary evaporator.

Cancer induction

Procedure for the Air pouch technique

By using the Arun et al. (1984) approach, air pouches were created in Wistar rats. The 5 ml syringe has around 2 ml of air poured into it. It underwent a 20-minute sealed autoclave at 15 psi. A sterile air pouch was created by gently injecting the sterile air from the syringe right beneath the mammary fat pad. Before giving the carcinogen, the air within the pouch was given a day to stabilise.

Application of a carcinogen

A sterile vial containing 20 mg of B(a) P was weighed, and then 0.5 ml of sterile saline and 0.5 ml of sunflower oil were added. To create an emulsion that was evenly spread, the vial was stoppered and aggressively vortexed. B(a)P was injected into the air pouch in a single dosage. Until the 90th day, when it reached its maximum size, the development of the tumour was monitored at regular intervals.

Organ and blood collection

Following the experiment, all test rats were executed via cervical decapitation. Ethylene diamine tetraacetic acid (EDTA) was used during blood collection to separate serum and plasma before measuring blood parameters. To get a 10% homogenate, the liver and breast tissues were homogenised using a motor-driven, teflon-coated homogenizer in a 0.1M Tris-HCl buffer at a pH of 7.4..

RESULTS

Compared to breast cancer-bearing animals treated with either Withaferin A or Propolis alone, the administration of Withaferin A and Propolis together effectively suppressed breast cancer as evidenced by a decrease in the extent of lipid peroxidation (LPO) and a concurrent increase in the activities of antioxidants.

Plasma & breast tissues of both control and in experimental animals Lipid peroxidation was measured by estimating the Malondialdehyde (MDA) concentration as nanomoles of MDA liberated/mg protein. It is found that the lipid peroxidation was significantly lower in the group -V, ($^{\#}p<0.001$), which is treated with both Propolis and WA in comparison with Group-II (cancer bearing animals). The groups-III & IV, treated alone with WA and Propolis respectively, show a slightly lower lipid peroxidation concentration than group -II animals, as shown in Table No.1.

Table no. 2 shows the effect of Withaferin A and propolis treatment on enzymatic and non-enzymatic antioxidants in the Breast tissues of control and experimental animals. The group-II cancer-bearing animals have a lower level of both enzymatic & non-enzymatic antioxidant levels in comparison with group-I. Group III & IV shows an increase of antioxidant levels when treated with WA & Propolis, respectively. A significant rise of both the enzymatic & non-enzymatic antioxidant levels($^{\#}p<0.001$) was found in Group-V compared to group-II, which was treated with both WA & Propolis.

Effects of Propolis and Withaferin A on Plasma Enzymatic Antioxidants in Control and Experimental Animals were shown in the figure:1. The cancer-bearing animal group shows a significantly lower concentration of enzymatic antioxidant levels in comparison with the control group. The cancer-bearing animals show an

Table 1. Withaferin A and propolis' impact on lipid peroxidation in experimental and control animals

Particulars	Group I	Group II	Group III	Group IV	Group V
Plasma	1.08±0.12	1.65±0.16a#	1.13±0.11b#	1.42±0.14b@	1.43±0.14b#
Breast	1.08±0.11	2.98±0.26a#	2.04±0.21b#	2.45±0.23b#	1.61±0.11b#

All the values are expressed in mean ± SD for all the six rats in each group.Units: Plasma: nmoles of MDA liberated/ mg protein; Breast: nmoles of MDA liberated/mg proteina - as compared with Group Ib - as compared with Group IIStatistical significance - $^{\#}p<0.001$, $^{@}p<0.01$, *p<0.05, NS -Not significant.

Table 2. Propolis and withaferin A's effects on enzymatic and non-enzymatic antioxidants in the breast of experimental and control animals

Particulars	Group I	Group II	Group III	Group IV	Group V
SOD	7.15±0.65	4.44±0.37a#	5.71±0.52b#	5.21±0.44b*	6.68±0.57b#
CAT	37.01±3.49	21.33±2.01a#	27.08±2.60b@	25.06±2.39b*	32.46±3.05b#
GPx	5.36±0.45	2.79±0.26a#	3.80±0.36b#	3.30±0.28b*	4.72±0.41b#
GSH	4.86±0.39	2.43±0.11a#	3.82±0.14b@	3.03±0.27b@	4.27±0.35b#
Vitamin C	2.63±0.23	1.54±0.11a#	2.02±0.19b#	1.85±0.14b@	2.43±0.22b#
Vitamin E	7.35±0.63	4.46±0.35a#	5.63±0.51b#	5.17±0.42b*	6.34±0.54b#

All the values are expressed in mean ± SD for six rats in each group
a: as compared with Group I
b: as compared with Group II
Statistical significance - #p<0.001, @p<0.01, *p<0.05.

improvement in the treatment with WA & Propolis on all the three enzymatic antioxidants but show a significant rise ($^{\#}p<0.001$) of catalase activity than glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity. The remarkable changes were found in group -V ($^{\#}p<0.001$) when compared with Group-II, where the catalase, SOD & GPx activity was found better in group-V with combination treatment of WA & Propolis.

Figure:2 The impact of withaferin A and propolis on non-enzymatic antioxidants in the plasma of experimental and control animals. The plasma concentration of non-enzymatic antioxidant levels decreased in the breast cancer-bearing animal group when compared with the control group. A notable decreased level of Vitamin-C, Vitamin-E & reduced glutathione (GSH) ($^{\#}p<0.001$) were found in group-II animals, which clearly indicates the increased levels of free radicals and oxidative stress. An outstanding improvement was shown by group-V ($^{\#}p<0.001$) in comparison with group-II, which may be due to the combination therapy of WA & Propolis. All the three non-enzymatic antioxidants in plasma show a remarkable increase in Group-III & IV in individual treatment of WA & Propolis, respectively. The antioxidant levels of control group and the group which received both WA & Propolis shows a significant relationship.

DISCUSSION

Oxidative stress increases in cancerous conditions by the increased free radical formation and cell proliferation. The antioxidant levels can be regained naturally by supplement treatment or by metabolically. The treatment with propolis and withaferin A shows remarkable progress in the antioxidant levels in the cancerous condition, but their combination therapy shows a significant increase in antioxidant levels. The study by Darvishi N and colleagues (2020) says, the propolis group showed a significant improvement in Pro-Oxidant-Antioxidant Balance (PAB).[13] A study done in Poland with extracts of Polish Propolis has demonstrated that they are rich in phenolic compounds and are very effective as antioxidant agents.[14] Another study about propolis reported that it may protect normal cells from radiation-induced oxidative stress in cancer radiotherapy, and it is recommended to use propolis with

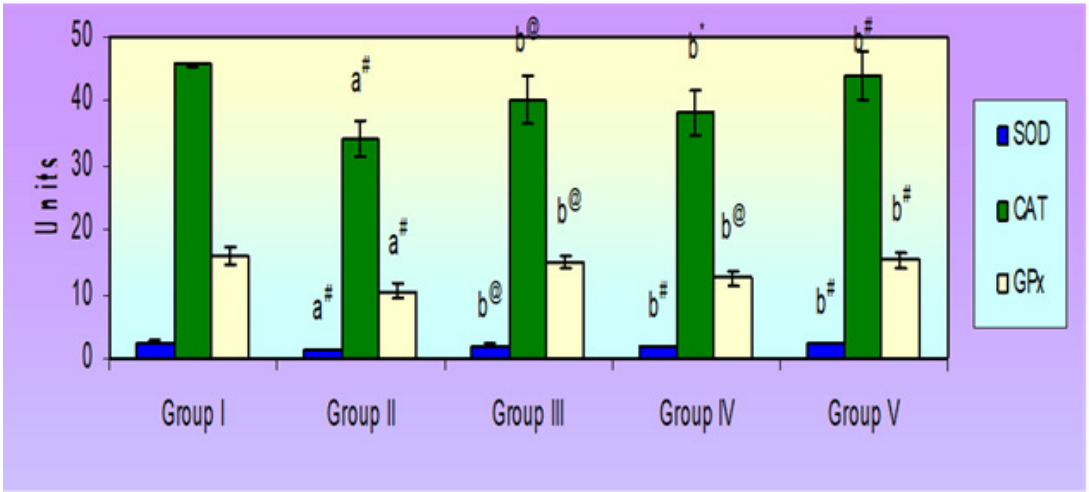

Fig. 1. Effects of Propolis and Withaferin A on Plasma Enzymatic Antioxidants in Control and Experimental Animals

Note: Each value is expressed as mean±SD for six rats in each group
SOD - units/min/mg protein; GPx - mmoles of H2O2 liberated/min/mg protein;
CAT - mmoles of H2O2 liberated/min/mg protein
a: as compared with Group I;
b: as compared with Group II
Statistical significance - #p<0.001, @p<0.01, *p<0.05

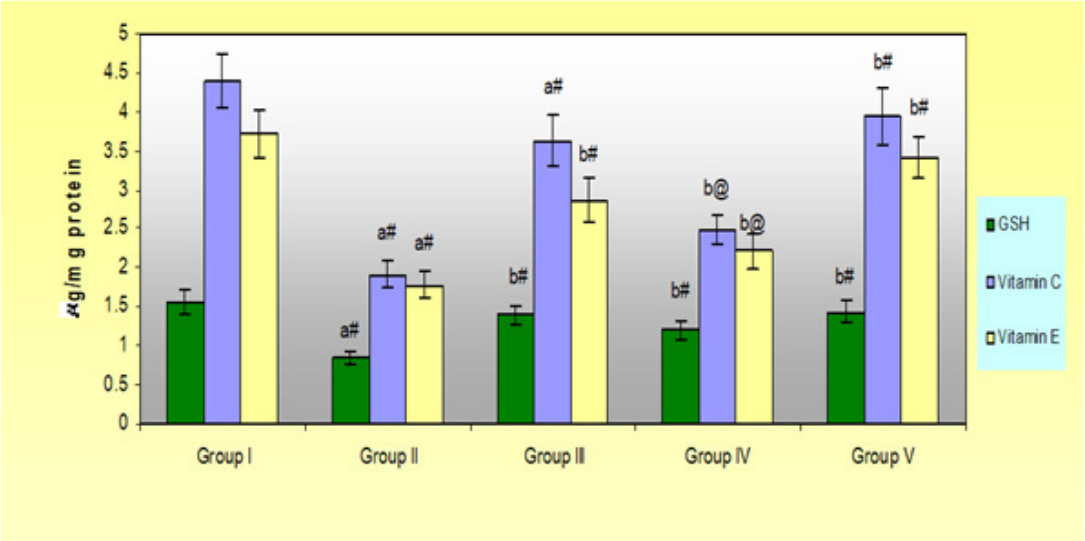

Fig. 2. The impact of withaferin A and propolis on non-enzymatic antioxidants in the plasma of experimental and control animals

Note: Each value is expressed as mean±SD for six rats in each group
a: as compared with Group I
b: as compared with Group II
Statistical significance - $^{\#}p<0.001$, $^{@}p<0.01$, $^{*}p<0.05$.

radiotherapy.[15] Another experimental study shows the increased activity of both SOD and CAT enzymes in mammary carcinoma of mice. It may be due to the properties of Withania roots to inhibit the formation of free radicals, increase termination of reactive oxygen species, or both.[16] Formation of free radicals increases in a bulk concentration which is difficult to manage and can induce biomolecule damage and which leads to the formation of lipid peroxidation and damage to the DNAs. As a result of lipid peroxidation end product, MDA is accumulated in the body as age increases and can be a reason for the formation of pathological conditions like cancers[17]. If a sufficient concentration of antioxidants is present in our body, it can benefit from controlling our body's defence system. Another study says A mono-functional inducer which increases the enzymatic antioxidant activity is present in withaferin extracts[18]. The abundance of free radical species in various diseases is regulated by the cellular antioxidant enzyme (SOD, CAT, and GPX) activities.[19-22]

Thus, the balance between the rate of generation of radicals and the scavenging of radicals is maintained as an essential part of biological homeostasis. Clinical trials and more studies are required to start practicing these natural sources as a treatment for various diseases.

CONCLUSION

Antioxidants like enzymatic (CAT, SOD & GPx) and non-enzymatic (reduced glutathione, vitamin E & vitamin C) antioxidants were measured in all the study group animals. The groups treated with Withaferin A, propolis alone showed improvement. However, the combination study of Withaferin-A & Propolis showed a better chemotherapeutic efficacy than the individual treatment. From this study, we conclude that the combination therapy with both withaferin A and Propolis may help increase the antioxidant levels, reduce breast cancer symptoms and restore the architecture of the mammary gland.

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Author's contribution

Dr. N Muninathan contributed in designing the study, development of the protocol, conducted the research and data collection and authored the article. Mrs. Meghalatha T S conducted the research and data collection, intellectual content, and authored the article. Dr. Suresh contributed to conduct the research and authored the article.

Conflicts of Interest

There is no conflicts of interest.

Funding Sources

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