

The antioxidant and antimutagenic activities of Ankaferd blood stopper, a natural hemostatic agent used in dentistry

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Background/aim: This study investigated the antioxidant and antimutagenic properties of Ankaferd blood stopper (ABS), a plant-based topical hemostatic agent used in Turkey to treat external hemorrhages and bleeding during dental surgery. While previous studies have examined the antimicrobial, antiinflammatory, and anticarcinogenic properties of ABS, to our knowledge, this is the first study to report on the antioxidant and antimutagenic activities of this drug.

Materials and methods: Antioxidant activity was evaluated using DPPH radical-scavenging and β -carotene-linoleic acid tests. Antimutagenic activity was assessed using the Ames *Salmonella*/microsome mutagenicity test with the bacterial mutant strains *Salmonella typhimurium* TA98 and TA100.

Results: Although ABS demonstrated no free-radical-scavenging activity in DPPH assays at the tested concentrations, β -carotene-linoleic acid testing found ABS to have a total antioxidant activity rate of $47.06 \pm 4.41\%$. Antimutagenic effects were observed on TA100 at plate concentrations of 5%, 0.5%, and 0.05%, and on TA98 only at a plate concentration of 5%.

Conclusion: ABS was shown to possess antioxidant and antimutagenic properties that could be of potential value in the fields of medicine and dentistry.

Key words: Ankaferd, antioxidant, Ames test, *Salmonella typhimurium*

1. Introduction

Mutagenicity refers to the induction of permanent transmissible changes in the structure of genetic material within cells and organisms (1). Mutations are caused by mutagens such as reactive oxygen species (ROS), ultraviolet radiation, and ionizing radiation and pollution, and are also the end products of normal metabolic processes of aerobic organisms (2–5).

Oxidative stress caused by ROS is known to cause tissue injury and can include damage to DNA, proteins, and lipids (6,7). Oxidative injury to DNA occurs when oxygen radicals react with DNA (8). If not repaired, the changes in nucleic acid bases and the breaks in the DNA chain that occur after free radical reactions lead to DNA mutation and mutagenic forms of DNA (9).

Cancer and degenerative diseases have been connected with the generation of excess ROS, inducing cell damage due to an imbalance between antioxidants and oxidants (10,11). Mutation is another important factor in

carcinogenesis (12). Mutagenicity and carcinogenicity are clearly correlated. One study showed that 157 of 175 known carcinogens (approximately 90%) are also mutagens (13).

Cancer is the second-leading cause of human death worldwide, despite many therapeutic measures being taken to control it (14). Oral cancer is a devastating disease that ranks as the fifth-most-common type of cancer affecting humans worldwide (15). The disease affects the mouth and pharynx and can include cancers of the lips, tongue, lower and upper palate, gingiva, alveolar and buccal mucosa, oropharynx, tonsils, uvula, and salivary glands. Oral squamous cell carcinoma alone represents the fifth-highest-ranking cancer, comprising 90% of all intraoral cancers worldwide (16,17).

Kada et al. (18) classified antimutagens according to two major groups: bioantimutagens and desmutagens. Whereas bioantimutagens modulate DNA replication and repair in order to prevent premutagenic lesions from transforming into mutations, desmutagens, including

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antioxidants, inhibit the conversion of promutagens into mutagens, inactivate mutagens, and prevent mutagen interaction with DNA (19,20).

Antioxidants, in particular those from natural sources, are the subject of extensive research due to their potentially important role in chemoprevention of cancer and other human degenerative disorders (19,20). Antioxidants are capable of stabilizing or deactivating free radicals, often before they attack intracellular targets (21). Both exogenous and endogenous antioxidants, whether synthetic or natural, can effectively scavenge free radicals or promote their decomposition, suppressing such disorders (22–25). Natural antioxidants have become the target of a great number of research studies aimed at finding sources of potentially safe, effective, and cheap antioxidants (26). The control of cellular mutability by natural antimutagens can help prevent the mutations that can conceivably result in cancer and other diseases caused by genotoxic agents (27,28).

In recent years, there has been increasing interest in investigating compounds originating from plants and their effects on DNA (29). Plant-based compounds could act as protective agents against the initiation, promotion, or progression of human carcinogenesis (30), or, perhaps, destroy or block DNA-damaging mutagens outside cells, thus preventing cell mutation (31).

Ankaferd blood stopper (ABS) (Ankaferd Health Products Ltd., Turkey) is a novel topical hemostatic agent developed from plant extracts used in traditional folk medicine in Turkey. Comprising a standardized mixture of *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica* (32), ABS has been approved by the Turkish Ministry of Health for the management of dental bleeding. It is included among the protocols for prevention and treatment of exaggerated hemorrhage related to dental procedures (33) and is indicated in surgical procedures when conventional bleeding control is ineffective (34).

The literature has reported on the use of ABS in experimental studies (35,36), gastrointestinal bleeding (37–42), urologic surgery (36,43), tonsillectomy (44), and acute anterior epistaxis (45). According to these studies, ABS seems to be effective as a topical hemostatic agent whose hemostatic effect involves the formation of an encapsulated protein network representing focal points for vital erythrocyte aggregation (34).

Studies have shown that each plant found in ABS has some effect on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics, or cell mediators (46–51). A study by Goker et al. (52) reported on the therapeutic potential of ABS in the management of hemorrhage, and several studies have reported on the antimicrobial (53–58), antifungal (59), antiinflammatory

(60), and anticancer (61,62) activity of ABS. However, no studies have examined the antioxidant and antimutagenic activity of ABS.

The objective of this study was to evaluate the antioxidant activity and antimutagenic potential of ABS, especially in terms of protection against mutations that cause oral cancer.

2. Materials and methods

2.1. ABS

ABS is a patented product (Turkish Patent No. 2007-0-114485) comprising a standardized mixture of the plants *G. glabra* (90 µg/mL), *V. vinifera* (80 µg/mL), *U. dioica* (60 µg/mL), *T. vulgaris* (50 µg/mL), and *A. officinarum* (70 µg/mL) (63,64). A 2-mL vial of ABS was obtained from the manufacturer (Trend Teknoloji İlaç AS, Turkey). Diluted ethanol solutions of 5% (1/20), 0.5% (1/200), and 0.05% (1/2.000) were used in the mutagenicity and antimutagenicity tests, whereas 100% (1/1), 50% (1/2), 25% (1/4), and 12.5% (1/8) solutions were used in the antioxidant tests.

2.2. Microbial strains

The mutagenicity and antimutagenicity tests were performed with an Ames *Salmonella*/microsome mutagenicity assay. The Ames test employs several histidine-dependent *Salmonella* strains, each carrying different mutations in various genes in the histidine operon (65). In this study the mutant strains *S. typhimurium* TA98 and TA100 were used. Both strains were analyzed according to Mortelmans and Zeiger (65) for histidine and biotin requirements alone and in combination, rfa mutation, excision repair capability, presence of plasmid pKM101, and spontaneous mutation rates. Bacterial stock cultures were inoculated in nutrient broth and incubated at 37 °C for 12–16 h with gentle agitation (66).

2.3. Antioxidant activity

2.3.1. Determination of DPPH radical scavenging activity

The antioxidant activity of the extracts was determined based on their ability to react with the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (67). Fifty microliters of ABS (100%, 50%, 25%, and 12.5% concentrations in ethanol) was added to 5 mL of DPPH solution (0.004%) in ethanol. After incubation at room temperature for 30 min, the absorbance of each solution was determined at 517 nm. The percentage of inhibition and the concentration of the sample required for 50% scavenging of the DPPH free radical (IC_{50}) were determined. Ascorbic acid and α -tocopherol were used as positive controls.

2.3.2. Total antioxidant activity by the β -carotene-linoleic acid method

The total antioxidant activity of the ABS was evaluated by the β -carotene-linoleic acid model (68). First 0.5 mg of

the β-carotene in 1 mL of chloroform, 25 μL of linoleic acid, and 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed together. The chloroform was completely evaporated using a vacuum evaporator and the resulting solution was diluted with 100 mL of oxygenated water. Next, 2.5 mL aliquots of this mixture were transferred into different tubes containing 0.5 mL of ABS (100% concentration). The same procedure was repeated with the positive control ascorbic acid, α-tocopherol, and a blank. The emulsion system was incubated for up to 2 h at 50 °C. Absorbance measurement was continued until the color of β-carotene disappeared in the control. After this incubation period, the absorbance of the mixtures was measured at 490 nm. All determinations were performed in triplicate.

The bleaching rate (R) of β-carotene was calculated using the following formula: $R = \ln(a/b)/t$, where \ln = natural log, a = absorbance at time 0, b = absorbance at time t (120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using the formula $AA = [(R_{Control} - R_{Sample})/R_{Control}] \times 100$. The AA of the extract was compared with those of ascorbic acid and α-tocopherol at 5 and 1 mg mL⁻¹, respectively.

2.4. Mutagenic and antimutagenic activity

2.4.1. Viability assays and determination of test concentrations

Cytotoxic doses of the ABS were determined according to Mortelmans and Zeiger (65). The toxicity of the ABS toward *S. typhimurium* TA98 and TA100 was determined as described in detail elsewhere (69,70).

2.4.2. Mutagenicity and antimutagenicity tests

The mutagenicity and antimutagenicity of ABS were examined using the plate incorporation method (71) described in detail by Sarac and Sen (72). Known mutagens 4-nitro-o-phenylenediamine (4-NPD) 3 μg/plate and sodium azide (NaN₃) (8 μg/plate) were used as positive controls for *S. typhimurium* TA98 and *S. typhimurium* TA100, respectively. Ethanol was used as a negative control. The ABS was used at the subcytotoxic doses (5%, 0.5%, and 0.05% concentrations of ABS/plate). Mutagenicity inhibition (%) was calculated using the following equation:

$$\text{Inhibition} = [(M - S_1)/(M - S_0)] \times 100$$

Where M = number of revertants/plate induced by mutagen alone;

S₀ = number of spontaneous revertants; and

S₁ = number of revertants/plate induced by the ABS plus the mutagen.

Antimutagenicity was recorded as follows: Strong: 40% or more inhibition; Moderate: 25%–40% inhibition; Low/None: 25% or less inhibition (27,73).

2.5. Statistical analysis

Experiments were performed in triplicate, and results were recorded as mean ± SD. Data were entered into a Microsoft Excel database and analyzed using SPSS.

3. Results

This study investigated the antioxidant and antimutagenic activities of ABS, a novel topical hemostatic agent used in Turkey to control external hemorrhaging and bleeding during dental surgery.

The free-radical-scavenging capacity of ABS was evaluated by DPPH assay. ABS did not demonstrate any radical scavenging activity at the tested concentrations (data not shown). Total antioxidant activity of ABS was evaluated using the β-carotene-linoleic acid method (Table 1). The results showed ABS to have a moderate level of total antioxidant activity that was lower than that of both ascorbic acid and α-tocopherol.

ABS tested at plate concentrations of 5%, 0.5%, and 0.05% showed no mutagenic effects on *S. typhimurium* TA98 or TA100 (data not shown). ABS antimutagenicity was assessed using the Ames test. The effects of 5%, 0.5%, and 0.05% ABS/plate concentrations were tested against 4-NPD on *S. typhimurium* TA98 and against NaN₃ on TA100 (Table 2). All the tested concentrations were effective on TA 100; however, only the 5% concentration was effective on TA 98. In general, the antimutagenic activity of ABS was found to be dose-dependent, with antimutagenicity increasing in line with increases in ABS concentrations. ABS showed strong antimutagenic activity at the higher test concentrations, with the strongest activity observed at 5% concentration on *S. typhimurium* TA 100.

4. Discussion

DNA mutation and cell- and tissue-level damage may occur as a result of ROS oxidation of DNA, lipids, proteins, carbohydrates, and other biological molecules (74,75). Natural antioxidants present in herbs and spices are known to prevent or at least inhibit the deleterious consequences of this oxidative stress (76).

Table 1. Antioxidant activity (%) of ABS in the β-carotene-linoleic acid test system.

Sample	Antioxidant activity (%)
ABS	47.06 ± 4.4 ^a
Ascorbic acid	60.63 ± 0.16
α-tocopherol	96.42 ± 2.60

^aValues expressed are means ± SD of three parallel measurements.

Table 2. The antimutagenicity assay results of the ABS for *S. typhimurium* TA98 and TA100 bacterial strains.

Test items	Concentration	Number of revertants			
		TA98		TA100	
		Mean ± SD	Inhibition%	Mean ± SD	Inhibition%
Negative control		7 ± 3.6 ^a		64.66 ± 8.62	
4-NPD*	3 µg/plate	346.66 ± 18.55		-	
NaN ₃ *	8 µg/plate	-		529.5 ± 65.56	
ABS	5%	217 ± 8.18	38.17	290 ± 12.96	51.52
	0.5%	310.33 ± 26.83	10.69	313.5 ± 19.22	46.46
	0.05%	318.66 ± 43.40	8.24	339.25 ± 11.72	40.92

*4-NPD and NaN₃ were used as positive controls for *S. typhimurium* TA98 and TA100 strains, respectively. ^aValues expressed are means ± SD of three parallel measurements. The regression analysis was carried out in Microsoft Excel between percent inhibition of mutagenicity and log values of concentrations of the ABS.

Mutations cause inborn errors of metabolism leading to morbidity and mortality in living organisms. The best way for humans to decrease the rate of mutation is to avoid the risk of mutation through exposure to or ingestion of mutagens and carcinogens (12). Mutations are the cause of inherited metabolic disorders as well as a spectrum of age-related human diseases, including cancer (77); thus, reducing mutation rates may reduce the incidence of cancer.

The Ames test is a short-term, reverse-mutation test used world-wide specifically to screen new chemical substances and drugs that could cause genetic damage and subsequent mutation (65). The *Salmonella* strains used in the test have mutations on a number of genes in the histidine operon, with each mutation designed to respond to mutagens operating via a different mechanism (65,71).

The most effective way of preventing cancer and genetic diseases in humans is through the use of antimutagens and

anticarcinogens in daily life (12). Natural antimutagens may control cellular mutability, preventing genotoxic agents from initiating mutations that could conceivably result in cancer and other diseases (27,28), and while the antimutagenicity of a plant extract is not a definitive indication of its anticarcinogenicity, it is certainly a sign of anticarcinogenic potential (78).

This study found ABS to exhibit antimutagenic and antioxidant activity in vitro. Considering that ABS was found to be safe at the tested concentrations, the results of this study suggest that ABS may represent a readily accessible source of natural material with a variety of applications, especially in the field of dentistry. Moreover, the antioxidant and antimutagenic properties of ABS indicate that its use as a topical hemostatic agent may provide prophylaxis against various diseases, including heart disease, stroke, arteriosclerosis, and cancer.

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