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Research Paper

Human epithelial carcinoma cytotoxicity and inhibition of DMBA/TPA induced squamous cell carcinoma in Balb/c mice by *Acacia catechu* heartwood

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Abstract

Objectives *Acacia catechu* heartwood contains significant amounts of polyphenolic compounds that exhibit powerful antioxidant activity. The purpose of this study was to evaluate the cytotoxicity of *A. catechu* heartwood extracts in a human epithelial carcinoma cell line (A431) and antitumour activity against DMBA/TPA induced squamous cell carcinoma in Balb/c mice.

Methods Various extracts, including aqueous, ethyl acetate, chloroform and *n*-hexane, were tested for cytotoxic properties on a human epithelial carcinoma cell line (A431) by using MTT, sulforhodamine B and lactate dehydrogenase leakage assays. The standardized *A. catechu* heartwood aqueous extract (AQCE) was further evaluated for antitumour activity against 7,12-dimethylbenz[a]anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced skin carcinoma in Balb/c mice.

Key findings The results showed that administration of AQCE showed a dose-dependent growth inhibition response, with an IC50 value of 78.56 µg/ml. Tumour incidence was significantly decreased ($P < 0.001$) to 30% with AQCE compared with 100% in the DMBA/TPA group. The AQCE was also found to significantly upregulate different antioxidant enzymes in skin and liver tissue.

Conclusions The results suggest that AQCE may exert its chemopreventive activity by acting as an antioxidant.

Keywords *Acacia catechu*; A431 cell line; antioxidant; cytotoxicity; squamous cell carcinoma

Introduction

Chemoprevention and the treatment of skin cancer are of paramount importance. Multidisciplinary scientific investigations are making the best efforts to combat this disease but the perfect cure has yet to be found. In spite of the advances made in basic scientific knowledge as well as clinical treatment of skin cancer, the death rate continues to rise.[1] Furthermore, most of the cytotoxic drugs used presently in skin cancer therapy are highly toxic to a wide range of tissues (e.g. gastrointestinal tract, bone marrow, heart, lungs, kidney and brain) and iatrogenic failure of these organs has been observed to be a frequent cause of death from cancer.[2] In the last few years there has been growing interest in the pharmacological evaluation of various plant products for the chemopreventive treatment of skin cancer. The links between dietary and environmental factors and the incidence of various cancers have been evaluated and there is increasing evidence that some constituents of plants found in the diet prevent damage to cells or other factors in its metabolism and function that predispose people to skin cancer. Many herbs have been evaluated in clinical studies and are currently undergoing phytochemical investigation to determine their tumouricidal action against various cancers. In spite of these successes, the magnitude and severity of the cancer problem make it imperative to develop a preventive approach to this disease.

Acacia catechu Willd (Cutch tree) belonging to the family Leguminosae is commonly used by traditional healers in herbal preparations as a thermogenic, digestive, appetizer, aphrodisiac, hepatoprotective, haemostatic, anthelmintic, depurative and tonic agent. It is also used for toothache, ulcerations and soreness of gums,[3] and asthma and bronchitis.[4] It has been reported to possess antipyretic, antidiarrhoeal, hypoglycaemic (wood), hepatoprotective,[5] anthelmintic,[6] antimicrobial[7] and antioxidant[8] activity. To date, however, there is

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no scientific evidence to demonstrate the anticancer activity (squamous cell carcinoma of skin) of *A. catechu* heartwood. Given its potential antioxidant activity and the role of antioxidants in chemoprevention of cancer, the present study was designed to evaluate its cytotoxicity in a human epithelial carcinoma cell line (A431) and its chemopreventive efficacy in a two-stage carcinogenesis model in mice.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulforhodamine B (SRB), 7,12-dimethylbenz (a)anthracene (DMBA), 12-*O*-tetradacanoylphorbol-13-acetate (TPA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), ethylene diamine tetra acetic acid (EDTA), sodium dodecyl sulfate (SDS), thiobarbituric acid, bovine serum albumin (BSA), reduced glutathione (GSH), yeast glutathione reductase, oxidized glutathione (GSSG), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were all obtained from Sigma Chemical Co. (St Louis, MO, USA). The lactate dehydrogenase (LDH) assay kit was purchased from Promega (Madison, WI, USA). Hydrogen peroxide 30% (H_2O_2) was obtained from Merck (Mumbai, India). The rest of the chemicals used were obtained from local firms and were of analytical grade.

Preparation of *A. catechu* heartwood extracts

A. catechu heartwood was collected from Hamirpur, Himachal Pradesh, India, and a voucher specimen (AC-2011) was deposited in the herbarium of the Biotechnology Department, Jaypee University of Information Technology, Waknaghat, Solan, India.

The dried powder of *A. catechu* heartwood (1 kg) was put in an aluminium pot with 10 l of water and boiled for 5 h. It was then allowed to stand for 24 h. The extract was decanted and filtered through a fine muslin cloth to remove suspended materials. The filtrate was evaporated and the residue obtained was air dried to obtain a solid mass. The percentage yield (w/w) of the aqueous extract was 21.23. The powdered heartwood material was extracted using solvents of increasing polarity (*n*-hexane, chloroform and ethyl acetate) in a Soxhlet extraction apparatus. The solvent was completely removed under reduced pressure to obtain a solid mass. The percentage yield (w/w) of each extract was 1.17, 2.11 and 2.67, respectively.

Qualitative phytochemical screening of *A. catechu*

Phytochemical analysis for major phytoconstituents of the plant extracts was undertaken using standard qualitative methods.[9–12] The plant extracts were screened for the presence of biologically active compounds such as saponins, reducing sugars, tannins, flavonoids, terpenoids and alkaloids.

Saponins

About 0.5 g of each extract was boiled in 20 ml of distilled water in a water bath and filtered. Then, 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously to form a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of an emulsion.

Reducing sugars

A total of 5 ml of each extract was mixed in 5 ml of Fehling's A and B solution. A brick red coloured precipitate indicated the presence of reducing sugars.

Tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

Flavonoids

Each extract (0.5 g) was treated with dilute NaOH, followed by the addition of dilute HCl. Solubility and colour were noted. A yellow solution with NaOH that turns colourless with dilute HCl confirms flavonoids.

Terpenoids (Salkowski test)

Each extract (5 ml) was mixed with 2 ml of chloroform, and concentrated H_2S0_4 (3 ml) was carefully added to form a layer. The formation of a reddish brown colouration at the interface indicated a positive result for the presence of terpenoids.

Alkaloids

About 0.5 g of each sample was boiled with 5 ml of 2% HCl on a steam bath for 5 min. The mixture was allowed to cool and was then filtered. The filtrate was divided equally between three labelled test tubes. Portions of the filtrate (1.0 ml) were treated with two drops of the following reagents: Dragendroff's, Wagner's and Mayer's reagent. A reddish brown colouration with Dragendroff's and Wagner's reagents, and creamy white coloured precipitate with Mayer's reagent indicated the presence of alkaloid.

HPLC analysis of *A. catechu*

Preparation of the standard

The stock solution was prepared by dissolving the appropriate amount of standard (+)-catechin (~5.0 mg) in 7 ml of diluent (0.1% formic acid water and methanol, 3 : 7) with sonication for 15 min. The final volume of the solution was then diluted to 10 ml with the diluent at room temperature.

Sample preparation

A total of 50 mg of dried powered sample was accurately weighed and placed into a 100-ml volumetric flask, and ~70 ml of diluent (0.1% formic acid water and methanol, 3 : 7) was added. The sample was sonicated for 20 min (only for 70% methanol extraction) and allowed to cool to room temperature, and then filled to the full volume with the diluent. The extract was transferred to a centrifuge tube and centrifuged at 12 000 g for 2 min to obtain a clear solution which was filtered through a 0.22-µm filter. The injection volume was 20 µl.

Chromatographic conditions

Analysis of the extract was performed by high performance liquid chromatography (HPLC). The quantification of (+)-

catechin was carried by reverse phase HPLC (HPLC Waters 515) through a C18 (5.0 μm) 250 mm × 4.6 mm Waters spherisorb symmetry column fitted with a photodiode array detector (Waters 2996). The mobile phase consisted of 0.1% trifluoroacetic acid/acetonitrile (85 : 15, v/v). Before use, the mobile phase was degassed using an ultrasonic bath and filtered using 0.4-μm membrane filters. The column was eluted in the isocratic mode with a flow rate of 1.0 ml/min. The (+)-catechin was detected at an absorbance wavelength of 279 nm. The cycle time of analysis was 25 min at 30°C. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards procured from Sigma Aldrich.

Isolation of (+) catechin

A total of 1 kg of the dried powder of *A. catechu* heartwood was put in an aluminium pot with 10 l of water and boiled for 5 h. It was then allowed to stand for 24 h. The extract was decanted and filtered through a fine muslin cloth to remove suspended materials. The filtrate was evaporated and the residue obtained was air dried to obtain a solid mass (212 g). The yield of catechu was 21.2%. Solid mass (150 g) was added to a 5 l stainless steel beaker containing 1 l distilled water. It was boiled with constant stirring for complete dissolution and then filtered. It was then evaporated to 500 ml and allowed to stand for 24 h. The aqueous filtrate was rejected. The residue was dissolve in ethanol and filtered. The ethanolic solution was evaporated to dryness and the residue was dissolved in 500 ml hot water. It was allowed to stand for 24 h. The precipitate was filtered and dried in air (m.p. 95–6°C, yield 37.5 g, 25%). The process of re-crystallization from water was repeated three times. The IR spectra (υ_{max} (KBr)) showed bands at 2600–3400 (broad), 1620, 1520, 1470, 1380, 1280, 1240, 1150, 1120, 1080, 1020 and 820 cm^{-1}. The mass spectra showed maximum at 290 and minimum at 55. Other fragments were seen at 139, 138, 110, 152, 151 and 123. The molecular mass corresponding to 290 was observed. The ^{1}H-NMR spectra showed peaks at δ 4.56 (H-2, d, J(H-2, H-3a) 7.8 Hz), 4.00 (H-3, ddd, J(H-3a, H-4e) 5.58 Hz, J(H-3a, H-4a) 8.50 Hz, J(H-3a, H-2a) 7.80 Hz), 2.54 (H-4a, dd, J(H-4a, H-3a) 8.50 Hz, J(H-4a, H-4e) 16.10 Hz), 2.90 (H-4e, dd, J(H-4e, H-3a) 5.50 Hz, J(H-4e, H-4a) 16.10 Hz), 5.87 (H-6, d, J(H-6, H-8) 2.3 Hz), 6.01 (H-8, d, J(H-8, H-6) 2.3 Hz), 6.89 (H-2′, d, J(H-2′, H-6′) 1.95 Hz), 6.79 (H-5′, d, J(H-5′, H-6′) 8.07 Hz), 6.73 (H-6′, dd, J(H-6′, H-2′) 1.94 Hz, J(H-6′, H-5′) 8.19 Hz) and 8.00 (phenolic protons, m). The ^{13}C-NMR showed peaks at δ 27.7 (C-4), 66.3 (C-3), 80.9 (C-2), 93.9 (C-6), 95.1 (C-8), 114.5 (C-2), 115.1 (C-5) and 18.4 (C-6), and other aromatic carbons showed peaks at δ 99.1, 130.6, 144.6, 144.8, 155.3, 156.1 and 156.4. The spectral data confirmed the (+) catechin structure.

Cell culture

The epithelial carcinoma cell line (A431) was obtained from the National Centre for Cancer Science (Pune, India) and grown as a monolayer in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin. Cells were incubated at 37°C in an atmosphere of 5% CO_2. Stock solutions of all extracts were prepared in dimethylsulfoxide and solutions with different concentrations were obtained by diluting with culture medium. The final concentration of dimethylsulfoxide was less than 0.4% in all experiments. For 96-well plates, cell were seeded at approximately 1.5×10^4 cells per well. For 24-well plates, cells were seeded at approximately 4×10^4 cells per well.

Assay for cell proliferation

Trypan blue cytometry

The effects of different extracts on the growth curve of A431 cancer cells were determined by means of the trypan blue exclusion method.[13] Briefly, 4×10^4 cells per well were seeded in a 24-well plate and then treated with various concentrations of different extracts. The plate was incubated at 37°C and the number of cultured cells in the different wells was counted using a hemocytometer after staining with 0.4% trypan blue after 48 h to calculate the percentage viability. Cells were examined and counted under a light microscope at 10× (Olympus, Japan). Percentage cell viability was calculated by the formula:

$$\%\ \text{cell viability} = (\text{no. of viable cells (unstained cells)}/\text{total no. of cells (stained and unstained)}) \times 100$$

MTT assay

The cell viability of A431 cells was assessed by the MTT colorimetric assay,[14] which is based on the reduction of MTT by the mitochondrial succinate dehydrogenase of intact cells to a purple formazan product. Briefly, the adherent A431 cells were incubated in 96-well microtiter plates for 48 h. Following the addition of the test compounds, the plates were incubated for an additional 48 h. Control wells contained medium alone. Three replicate wells were used at each point in the experiments. After 48-h incubation at 37°C, MTT solution (5 mg/ml in phosphate-buffered saline) was added and incubated for another 4 h at 37°C in a 5% CO_2 incubator. The resulting MTT/formazan product was dissolved by 100 μl of isopropanol and the plates were gently shaken to solubilise the formed formazan. The amount of formazan was determined by measuring the absorbance (OD) at 570 nm using a Bio-Rad 550 enzyme-linked immunosorbent assay (ELISA) microplate reader. Cell survival was calculated as the percentage MTT inhibition as follows:

$$\%\ \text{growth inhibition} = 100 - (\text{mean OD of individual test group}/\text{mean OD of each control group}) \times 100.$$

SRB assay

Growth inhibition was determined using the SRB assay,[15,16] which estimates cell number indirectly by measuring total basic amino acids. The adherent A431 cells were incubated in 96-well microtiter plates for 48 h. Following the addition of the test compounds, the plates were incubated at 37°C for an additional 48 h in a 5% CO_2 incubator. The culture medium was then discarded and the cells were fixed *in situ* by the gentle addition of 100 μl of cold 10% (w/v) trichloroacetic acid and incubated for 60 min at 4°C. The supernatant was discarded and the plates were washed five times with tap

water and air dried. SRB solution (100 µl) at 0.4% (w/v) in 1% acetic acid was added and plates were incubated for 20 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilised with 10 mM Tris (pH 10.5) and the absorbance was read at 515 nm on a Bio-Rad 550 ELISA microplate reader.

LDH leakage assay

In the LDH assay,[17] leakage of the cytoplasmic located enzyme LDH into the extracellular medium is measured. The presence of the exclusively cytosolic enzyme, LDH, in the cell culture medium is indicative of cell membrane damage. For the LDH assay, 1.5×10^4 A431 cells per well were seeded in 96-well microtiter plates. At 48 h after cell seeding, cells were exposed to varying concentrations of the plant extracts. After 48 h of treatment, the supernatants were collected from each well. Cell monolayers were trypsinized and the cells and lysate were collected. LDH activity was measured in both the supernatant and the cell lysate fractions using Cyto-Tox 96, a non-radioactive cytotoxicity assay kit (Promega) in accordance with the manufacturer's instructions. The colour intensity is proportional to LDH activity. The absorbance was determined at 490 nm with a 96-well ELISA microplate reader. The percentage LDH release from the cells was determined using the following formula:

$$\text{LDH release} = \left(\left(\text{absorbance of the supernatant}\right)/\left(\text{absorbance of the supernatant and cell lysate}\right)\right) \times 100$$

In-vivo chemopreventive activity

Animals

Male Balb/c mice, 6–8 weeks old, 26 ± 2 g, were procured from the Animal House of B.N. College of Pharmacy, Udaipur, Rajasthan (CPCSEA no. 13/RKN/BNCP-06). They were kept in the departmental animal rooms with a controlled temperature of 23 ± 5°C, 60 ± 5% humidity and a 12-h light–dark cycle. They were fed a standard diet and water. The mice were acclimatized for 1 week before experimentation. The animal care and handling was done according to the guidelines set by the World Health Organization (Geneva, Switzerland) and the Indian National Science Academy (New Delhi, India). The protocol was approved by the institutional ethical committee for animal handling.

Experimental design and tumour induction

After 1 week of acclimatization, the mice were randomly divided into three groups ($n = 10$). Mice in group 1 served as controls. These animals received topical application of acetone (100 µl per mouse) only. For induction of skin tumours, groups 2 and 3 received topical application of DMBA (100 nmol/100 µl of acetone) for 2 weeks, followed by TPA (1.7 nmol/100 µl of acetone) 3 times a week for 20 weeks. Group 3 animals also received oral administration of 200 µl of AQCE at a dose of 400 mg/kg using a blunt tipped cannula 3 times a week throughout the experiment. AQCE was administered orally 1 week before the start of topical application of DMBA in Group 3. The numbers of lesions (papillomas as well as tumours) appearing on depilated skin were recorded at weekly intervals.

Tumour analysis

The incidence of skin papillomas, average number of papillomas per mouse, tumour volume, bodyweight, and number of animals that survived the 20-week period were recorded. The bodyweight, number of deaths, and papillomas appearing on depilated skin were recorded at weekly intervals. Only those papillomas that persisted for 2 weeks or more were taken into consideration for the final evaluation of the data. Mice were killed by cervical dislocation at the end of experiment. Tumour volume and burden were calculated as described by Subapriya and Nagini[18] using the following formulae.

$$\text{Mean tumour volume} = 4/3\pi r^3$$

where r is the mean tumour radius in mm.

$$\text{Mean tumour burden} = \text{mean tumour volume} \times \text{mean number of tumours}$$

Histopathological studies

Histopathological investigations were performed on the skin/tumourous tissues of the control as well as the treated animals. Tissues were fixed in 10% neutral buffered formalin for several days at room temperature. Fixed tissues were processed for paraffin-wax embedding, sectioned 4–5 μm thick and stained with hematoxylin and eosin. These slides were examined by pathologists at the Medicose Laboratory, Chandigarh, India, and at the Postgraduate Institute of Medical Education and Research, Chandigarh, India.

Biochemical parameters

The liver and dorsal skin tumours were quickly excised and washed thoroughly with chilled phosphate-buffered saline (pH 7.4). A 10% tissue homogenate (w/v), using a mechanically driven Teflon fitted potter Elveihem homogenizer, was prepared from part of the sample (liver/skin or tumours) in 0.15 M Tris-HCl (pH 7.4). Aliquots of tissue homogenate were kept at 4°C for the estimation of GSH. The remaining homogenate was subjected to cold centrifugation (4°C) at 10 000 *g* for 30 min. The supernatant obtained was used for the estimation of malondialdehyde (MDA), glutathione *S*-transferase (GST), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR).

Biochemical estimation

Estimation of skin and liver GSH

GSH was estimated as total non-protein sulfydryl groups according to the method described by Moron.[19] Homogenates (proteins) were immediately precipitated with 0.1 ml of 25% trichloroacetic acid and precipitates were removed after centrifugation at 15 000 *g* for 10 min to obtain the protein-free supernatant. Free SH groups were assayed in a total volume of 3 ml by adding 2 ml of 0.6 mM DTNB prepared in 0.2 M sodium phosphate buffer (pH 8.0), to 0.1 ml of

the supernatant, and absorbance was recorded at 412 nm using a double beam spectrophotometer. GSH was used as standard. The levels of GSH are expressed as nmol/mg protein.

Quantitative estimation of skin and liver lipid peroxidation

Levels of lipid peroxides were estimated using the method of Ohkawa *et al*.[20] Briefly, thiobarbituric acid (0.8%), SDS (0.1%) and acetic acid (20%) were added to 100 ml of the tissue homogenate (10%) prepared as described above. This mixture was heated for 30 min, cooled, extracted with *N*-butanol-pyridine, and the optical density of MDA recorded at 532 nm. The content of MDA is expressed as nmol/mg protein.

Estimation of skin and liver GST activity

The cytosolic or supernatant GST activity was measured spectrophotometrically at 37°C.[21] The reaction mixture (3 ml) contained 1.7 ml of 100 mM phosphate buffer (pH 6.5) and 0.1 ml of 30 mM CDNB. After pre-incubating the reaction mixture at 37°C for 5 min, the reaction was started by the addition of 0.1 ml diluted cytosol or supernatant and the absorbance was recorded at 340 nm. Reaction mixture without the enzyme was used as a control. The specific activity of GST is expressed as µmol of GSH-CDNB conjugate formed/min per mg protein using an extinction coefficient of 9.6 mM/cm.

Estimation of skin and liver CAT activity

This was assayed by the method of Aebi.[22] The change in absorbance was followed spectrophotometrically at 240 nm after the addition of H_2O_2 (30 mM) to 100 µl of the supernatant (10% tissue homogenate obtained as described above) in 50 mM phosphate buffer (pH 7.0). The activity of the enzyme is expressed as U/mg protein, where 1 IU is equivalent to 1 mol of H_2O_2/µg per min per mg protein.

Estimation of skin and liver SOD activity

SOD activity in liver and skin cytosol was assayed by the method Kono,[23] wherein reduction of nitroblue tetrazolium mediated by superoxide anions generated by photo-oxidation of hydroxylamine hydrochloride to blue formazon was measured at 560 nm. The activity of superoxide dismutase was expressed as IU/mg protein, where 1 IU is defined as the amount of enzyme inhibiting the increase in optical density by 50%.

GR activity

GR activity was determined by the procedure described by Carlberg and Mannervik.[24] Reaction mixture (final volume 1 ml) contained 0.2 M sodium phosphate buffer (pH 7.0), 2 mM EDTA, 1 mM oxidized glutathione (GSSG), and 1.2 mM NADPH. The reaction was started by adding 25 µl of 10 000 *g* supernatant and the enzyme activity was measured indirectly by monitoring the oxidation of NADPH following a decrease in OD/min for a minimum of 3 min at 340 nm. One unit of enzyme activity has been defined as nmol NADPH consumed/min per mg protein based on an extinction coefficient of 6.22 mM/cm.

GPx activity

GPx activity was measured by the coupled assay method as described by Paglia and Valentine.[25] Briefly, 1 ml of the reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) containing 1 mm EDTA, 0.24 U/ml yeast glutathione reductase, 0.3 mM glutathione (reduced), 0.2 mM NADPH, 1.5 mM H_2O_2, and 10 000 *g* supernatant. The reaction was initiated by adding NADPH and its oxidation was monitored at 340 nm by observing the decrease in OD/min for 3 min. One unit of enzyme activity has been defined as nmol of NADPH consumed/min based on an extinction coefficient of 6.22 mM/cm.

Statistical analysis

Data are expressed as means ± SEM. Statistical comparisons were performed by one-way analysis of variance followed by Tukey's test. In skin papillomagenesis, differences between control and experimental values were statistically analysed using the unpaired Student's *t*-test. Values of $P \leq 0.05$ were considered significant.

Results

Phytochemical analysis and isolation of (+) catechin

The results of the qualitative analysis of *A. catechu* heartwood are shown in Table 1. The results showed the presence of saponins, flavonoids, tannins and terpenoids, and the absence of alkaloids and reducing sugars. Tannins and flavonoids were present in the aqueous extract of *A. catechu* heartwood. Terpenoids were present in the ethyl acetate, *n*-hexane and chloroform extracts. Saponins were found only in the *n*-hexane extract.

The aqueous extract of *A. catechu* heartwood was analysed using a HPLC method, modified after Burnett *et al*.,[26] using reverse phase HPLC and the chromatographic profile was compared with the reference standard (Figure 1) obtained under the same conditions and the respective UV spectra. As (+)-catechin is known for its antioxidant

Table 1 Qualitative analysis of *Acacia catechu* heartwood

Sample no.	Extract	Saponins	Reducing sugars	Tannins	Flavonoids	Terpenoids	Alkaloids
1	Aqueous	–	–	+	+	–	–
2	Ethyl acetate	–	–	–	–	+	–
3	*n*-Hexane	+	–	–	–	+	–
4	Chloroform	–	–	–	–	+	–

–, Absent; +, present.

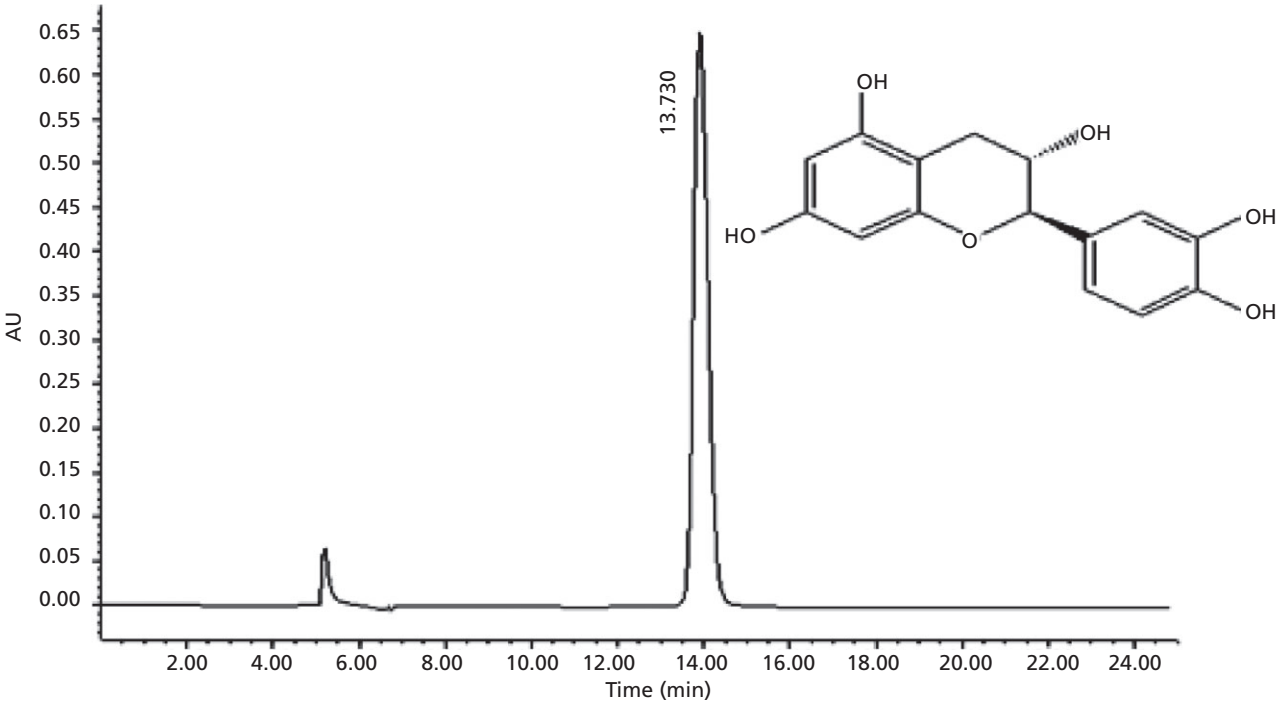

Figure 1 HPLC chromatogram of a standard sample of (+)-catechin.

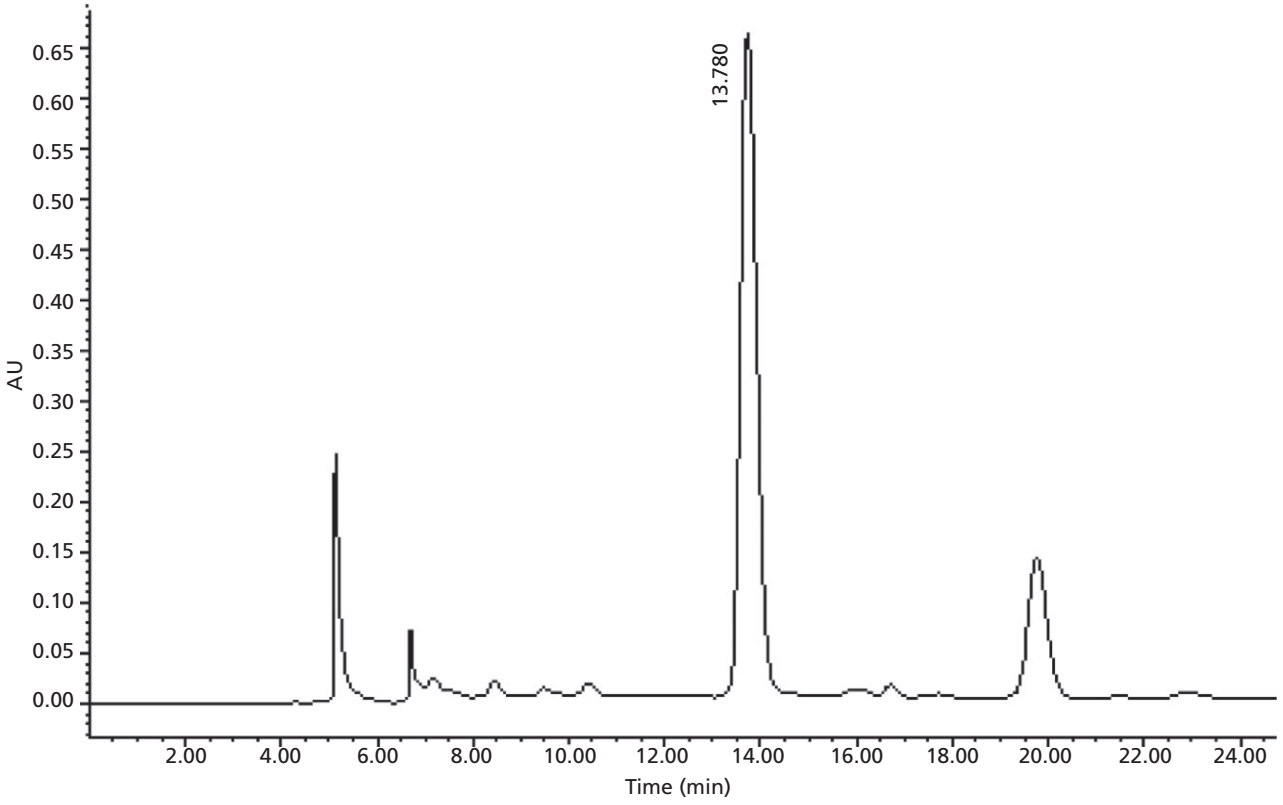

Figure 2 HPLC chromatogram of the aqueous extract of *Acacia catechu* heartwood.

Table 2 Effect of *Acacia catechu* heartwood extracts on percent cell viability (A431 cells) using the trypan blue exclusion assay

Concentration (μg/ml)	Percent cell viability			
	Aqueous extract	Ethyl acetate extract	Chloroform extract	*n*-Hexane extract
10	97.23 ± 0.33	99.03 ± 0.78	99.12 ± 0.13	99.21 ± 0.10
20	91.4 ± 0.19	96.11 ± 0.65	98.81 ± 0.10	98.93 ± 0.12
30	84.01 ± 0.28	95.29 ± 0.77	97.77 ± 0.42	97.81 ± 0.24
40	76.7 ± 0.77	93.13 ± 0.57	95.34 ± 0.39	96.10 ± 0.18
50	70.8 ± 0.44	90.8 ± 0.89	92.13 ± 0.61	93.16 ± 0.28
60	62.74 ± 0.49	86.23 ± 0.33	90.13 ± 0.47	91.74 ± 0.47
70	57.1 ± 0.36	81.12 ± 0.56	86.89 ± 0.46	88.13 ± 0.38
80	51.25 ± 0.41	76.41 ± 0.48	84.39 ± 0.24	85.29 ± 0.26
90	44.6 ± 0.68	73.18 ± 0.87	81.14 ± 0.17	82.63 ± 0.17
100	36.1 ± 0.38	70.37 ± 0.49	77.34 ± 0.14	79.11 ± 0.14

Each value represents the mean ± SEM of six replicates.

Table 3 Cytotoxicity (IC50) of catechu extracts on A431 cells

Extract	MTT	SRB	LDH
Aqueous	78.56 ± 0.55	80.52 ± 0.30	77.18 ± 0.75
Ethyl acetate	182.90 ± 0.70	183.53 ± 0.45	177.70 ± 0.50
Chloroform	223.86 ± 0.38	224.32 ± 0.79	221.6 ± 0.65
n-Hexane	242.23 ± 0.30	243.23 ± 0.44	240.2 ± 0.90

IC50 values (μg/ml) were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulforhodamine B (SRB) and lactate dehydrogenase (LDH) leakage assays. Each value represents mean ± SEM of six replicates.

properties, it was chosen as a bioactive marker for the standardization of the extracts. The (+)-catechin peak appeared at a retention time of 13.730 min (Figure 1). The mixture of 0.1% ortho phosphoric acid and acetonitrile gave optimum chromatographic separation of (+)-catechin and the other peaks in the extract (Figure 2). From the chromatographic profiles it was observed that the plant extract was rich in (+)-catechin (84.64%). The (+)-catechin peak did not interfere with any other peak in the extract samples, which indicated the specificity of the proposed method. (+)-Catechin was also isolated from the most active extract (aqueous) and characterized by spectral analysis.

In-vitro proliferation studies

In the present study, the cytotoxic effect of different catechu extracts on A431 cells were characterized by the trypan blue dye exclusion method and three different colorimetric assays (MTT, SRB and LDH release). Control assays were carried out for samples containing only the appropriate volumes of blank solutions and showed no effect on cell growth. A431 cells were exposed to different catechu extracts for about 48 h and cytotoxicity was determined with different cytotoxic assays. The percentage cell viability of different catechu extracts on A431 cells was evaluated by the trypan blue dye exclusion method. The results of trypan blue assay are presented in Table 2. IC50 values obtained by MTT, SRB and LDH assays for these incubation times are given in Table 3. The effect of different catechu extracts against the A431 cell line (concentration range 10–100 μg/ml) showed a decrease in percent cell viability in a dose-dependent manner, as compared with that of the control when examined by the trypan blue exclusion assay. Overall, the aqueous catechu extract showed the greatest activity. The MTT assay showed that the percentage growth inhibition increases with increasing concentrations of extracts steadily and linearly (from regression equation analysis study) for aqueous, ethyl acetate, chloroform and *n*-hexane extracts on A431 cells. The IC50 was calculated for all tested extracts from the statistical analysis (regression equation). The IC50 values for catechu extracts were 78.56, 182.90, 223.86 and 242.23 μg/ml, respectively, on A431 cells (Table 3). The IC50 values determined by the SRB method were 80.52, 183.53, 224.32 and 243.23 μg/ml for aqueous, ethyl acetate, chloroform and *n*-hexane extracts, respectively, on A431 cells.

Membrane integrity was evaluated by measuring LDH activity. The results obtained in the present in-vitro study indicated a significant increase in LDH release when the A431 cells were exposed to varying concentrations of AQCE. The results of the LDH assay are presented in Table 3. It was found that the percentage growth inhibition increased with increasing concentrations of compounds, steadily in the case of the aqueous extract against A431 cells, whereas with other extracts this linearity was not observed. The aqueous extract showed a good linearity range against A431 cells compared with the ethyl acetate, chloroform and *n*-hexane extracts.

The results showed that the aqueous extract was the most effective against human epidermis squamous cell line (A431) with a low IC50 value, followed by the ethyl acetate, chloroform and *n*-hexane extracts, respectively.

In-vivo studies

The present investigation indicated that the AQCE showed significant antitumour and antioxidant activity in DMBA/TPA treated animals. During the experimental period, the mice were observed at weekly intervals for changes in diet and water consumption, bodyweight, number of deaths, and papillomas appearing on depilated skin. Non-significant changes were observed in the diet and water consumption by the mice in all the groups studied as compared with their control counterparts (data not shown).

Effect of AQCE on DMBA/TPA-induced tumours in Balb/c mice

Tumour incidence was found to be 100% in the groups that received DMBA/TPA (Group 2). The mean tumour volume for mice in Group 2 was 553.1 mm^3. Upon treatment with AQCE (Group 3), it was found to decrease significantly ($P < 0.001$) to 194.53 mm^3. Similarly, the mean tumour burden was found to decrease significantly upon AQCE administration by 84.40% when Group 3 mice were compared with mice Group 2 mice (Table 4). The incidence of papillomas in DMBA/TPA treated and DMBA/TPA/AQCE treated groups at different weeks is shown in Figure 3. The onset of papillomas was observed at Week 7 (40%) in the DMBA/TPA treated mice (Group 2). There was a gradual rise in the incidence of cancer that reached 100% during Week 12. Mice in Group 3 that received AQCE treatment throughout the initiation stage of the experiment at a dose of 400 mg/kg showed a tumour incidence of 30%. The incidence of DMBA/TPA-induced papillomas was delayed by 4 weeks in the AQCE treated mice (Group 3). An overall significant decrease in incidence of cancer (30% versus 100%) was seen in the AQCE group at the end of Week 20 compared with DMBA alone.

The number of papillomas per papilloma-bearing mouse (papilloma yield) in the AQCE treated group was also significantly less ($P < 0.001$) than in the carcinogen control group at the end of the study. The average number of papillomas in AQCE treated mice (Group 3) was 2.66, whereas in DMBA treated animals it was 6.0 at the end of the study (Figure 4). Application of acetone alone on the depilated backs of mice did not induce papillomas throughout the experiment. The survival rate of mice decreased significantly in DBMA/TPA treated mice compared with the vehicle treated group. Survival of the AQCE treated group was significantly higher (70%) in comparison with the DMBA treated group (30%; Figure 5). The average bodyweight of DMBA/TPA treated mice (Group 2) did not differ from that of the acetone treated mice (Group 1) throughout the study. However, there was a slight increase in the average bodyweight of AQCE treated mice (Group 3) at the end of the study (Figure 6).

Histological investigations revealed normal skin and the presence of subcutaneous tissue in acetone treated mice

Table 4 Modulatory effect of the aqueous extract of *Acacia catechu* heartwood and/or DMBA/TPA on skin tumours of Balb/c mice

Parameter	Group 1	Group 2	Group 3
Mean tumour volume (mm^3)	NA	553.1	194.53***
% Reduction in mean tumour burden	NA	-	84.40%

Group 1: control; Group 2: DMBA/TPA; Group 3: DMBA/TPA/aqueous extract of *Acacia catechu*. NA, no tumour induction. ***$P < 0.001$, statistical significance: Group 3 versus Group 2.

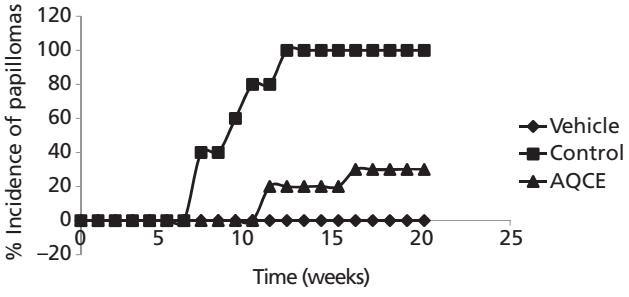

Figure 3 Effect of the aqueous extract of *Acacia catechu* heartwood (AQCE) on the percent incidence of papillomas after 20 weeks of DMBA/TPA application. Incidence was significantly reduced ($P < 0.001$) in the AQCE treated group compared with the corresponding values in the carcinogen control group.

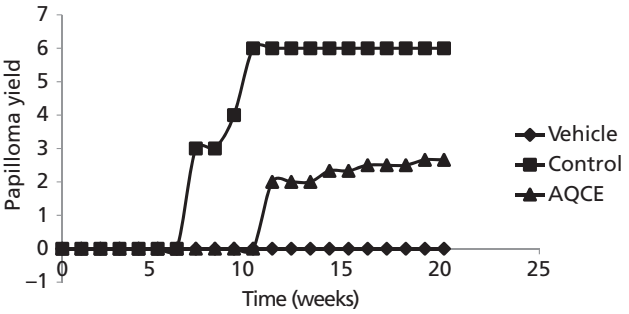

Figure 4 Effect of the aqueous extract of *Acacia catechu* heartwood (AQCE) on the average number of papillomas per mouse (papilloma yield) compared with the DMBA treated group. A significant reduction ($P < 0.001$) in the average number of papillomas was observed in AQCE treated group compared with the carcinogen control group.

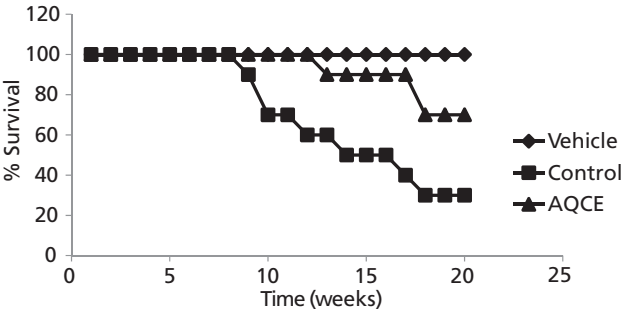

Figure 5 Effect of the aqueous extract of *Acacia catechu* heartwood (AQCE) on the percent survival rate of mice. $P < 0.002$ versus DMBA/TPA treated mice.

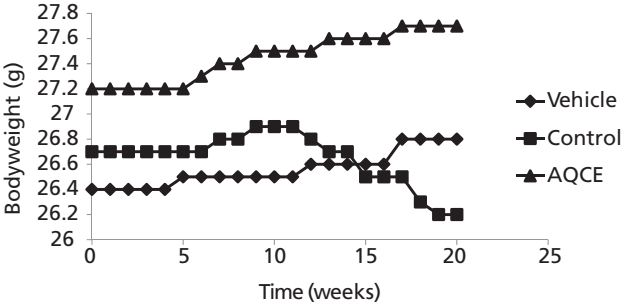

Figure 6 Effect of the aqueous extract of *Acacia catechu* heartwood (AQCE) on the average bodyweight of mice. $P < 0.01$ versus DMBA/TPA treated mice.

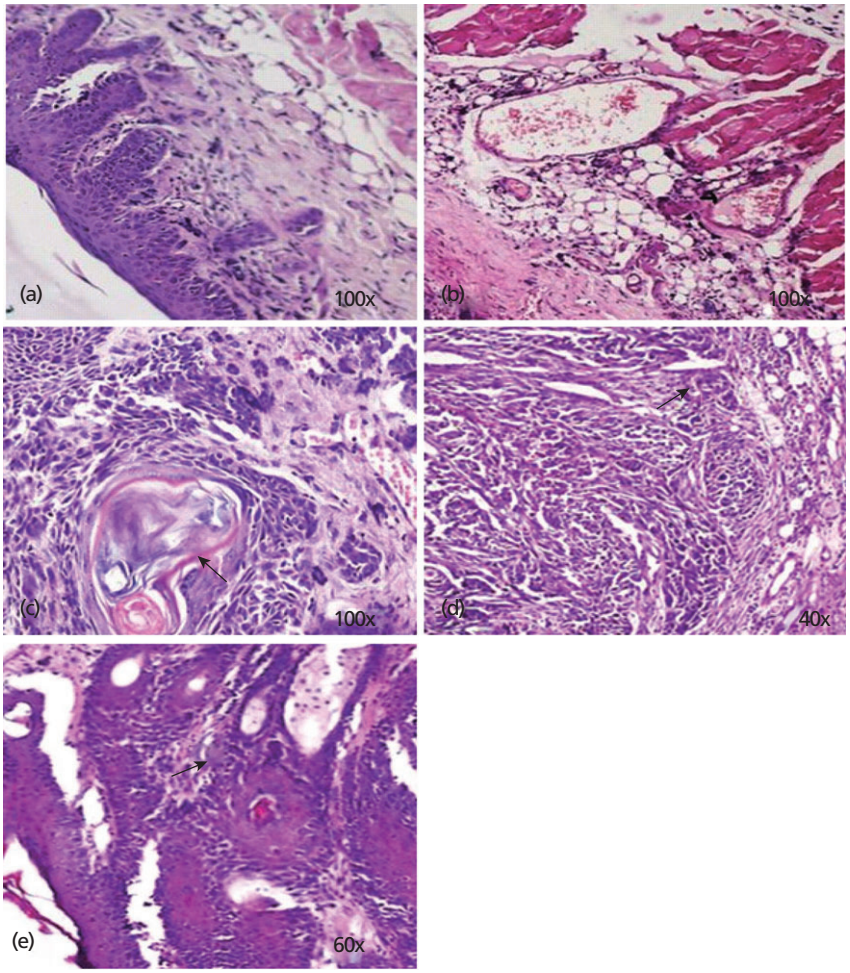

Figure 7 Hematoxylin and eosin stained sections of skin tumours of control, DMBA/TPA and DMBA/TPA/AQCE treated mice. (a) Hematoxylin and eosin stained section of control skin. (b) Squamous epithelial cells lying in the dermis clearly identify the tumours as invasive in the DMBA/TPA treated mice. (c) Characteristic squamous epithelial pearls. Also identifiable are the necrotic keratinocytes in tumours from DMBA/TPA treated mice. (d) Section showing the intact basal layer of tumours of DMBA/TPA/AQCE treated mice. (e) Hyperplastic lesions indicating the hyperplasia of the overlying epidermis in tumours of DMBA/TPA/AQCE treated mice.

(Group 1). The characteristic squamous pearls were clearly visible on examining the hematoxylin and eosin stained sections of the tumours formed on application of DMBA/TPA (Group 2). Islands of dysplastic squamous epithelial cells lying in the dermis distinctly indicated an invasive form of frank squamous cell carcinoma. Necrotic keratinocytes were also visible (Figure 7b and 7c). Tumours of Group 3 animals (DMBA/TPA/AQCE treated animals) displayed intact basement membrane with hyperplasia of the overlying epidermis. This clearly indicates the benign nature of these tumours (Figure 7d and 7e).

Biochemical studies in mouse skin tumour and liver

Effect on lipid peroxidation

The level of lipid peroxidation in the skin of mice treated with DMBA/TPA increased by 55.39% ($P < 0.001$), whereas mice treated with DMBA/TPA/AQCE exhibited only a 17.84% ($P < 0.01$) increase compared with vehicle treated control mice. In general, a 24.16% decrease ($P < 0.01$) in the level of MDA was observed in the tumours of mice treated with DMBA/TPA/AQCE in comparison with the MDA levels in the tumours of DMBA/TPA treated animals (Table 5). The MDA level increased in the liver by 143.56% ($P < 0.001$) in animals treated with DMBA/TPA, whereas animals treated with DMBA/TPA/AQCE exhibited an 82.95% increase ($P < 0.01$) in the MDA level in the liver compared with vehicle treated control animals. In other words, a 24.88% decrease ($P < 0.01$) in the liver MDA level of DMBA/TPA/AQCE treated animals was observed compared with the level in DMBA/TPA treated animals (Table 6).

Effect on GSH level

A 36.44% decrease ($P < 0.01$) in the levels of GSH in tumours of animals treated with DMBA/TPA, and a 15.81% decrease ($P < 0.05$) was observed in the tumours of animals treated with DMBA/TPA/AQCE compared with vehicle treated control animals (Table 5). There was an average 32.46%

Table 5 Effect of the aqueous extract of *Acacia catechu* heartwood on biochemical parameters in mouse skin tumours

Parameter	Group 1	Group 2	Group 3
MDA (nmol/mg protein)	2.13 ± 0.34	3.31 ± 0.26***	2.51 ± 0.24**
GSH (nmol/mg protein)	32.13 ± 1.2	20.42 ± 1.46**	27.5 ± 1.3*
CAT (U/mg protein)	7.8 ± 0.47	2.49 ± 0.5***	4.1 ± 0.56***
SOD (U/mg protein)	14.3 ± 1.17	5.6 ± 0.3***	9.8 ± 1.41**
GST (CDNB-GSH conjugate/min per mg protein)	31.3 ± 0.76	17.83 ± 1.23**	24.34 ± 1.03**
GR (nmol NADPH oxidized/min per mg protein)	4.6 ± 0.28	7.4 ± 0.30***	6.1 ± 0.25***
GPx (nmol NADPH oxidized/min per mg protein)	11.3 ± 1.7	21.3 ± 1.07**	16.1 ± 1.14**

Group 1: control; Group 2: DMBA/TPA; Group 3: DMBA/TPA/aqueous extract of *Acacia catechu*. MDA, malondialdehyde; GSH, reduced glutathione; CAT, catalase; SOD, superoxide dismutase; GST, glutathione *S*-transferase; GR, glutathione reductase; GPx, glutathione peroxidase. Data represent the means ± SEM in each group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle treated and DMBA/TPA or DMBA/TPA/AQCE. Comparisons were made between control (Group 1) and DMBA/TPA (Group 2) treated mice and between DMBA/TPA (Group 2) and DMBA/TPA/AQCE (Group 3) treated mice.

Table 6 Effect of the aqueous extract of *Acacia catechu* heartwood on biochemical parameters in mouse liver

Parameter	Group 1	Group 2	Group 3
MDA (nmol/mg protein)	2.64 ± 0.70	6.43 ± 0.36***	4.83 ± 0.66**
GSH (nmol/mg protein)	98.3 ± 1.29	24.9 ± 1.08***	60.13 ± 0.8***
CAT (U/mg protein)	6.13 ± 0.27	4.12 ± 0.5**	5.8 ± 0.40*
SOD (U/mg protein)	8.14 ± 1.82	3.39 ± 1.10**	6.47 ± 0.8**
GST (CDNB-GSH conjugate/min per mg protein)	54.2 ± 0.5	23.9 ± 1.19***	37.1 ± 1.07**
GR (nmol NADPH oxidized/min per mg protein)	6.4 ± 0.20	11.9 ± 0.10***	8.9 ± 0.40**
GPx (nmol NADPH oxidized/min per mg protein)	13.2 ± 1.9	24.9 ± 2.10**	19.2 ± 1.30**

Group 1: control; Group 2: DMBA/TPA; Group 3: DMBA/TPA/aqueous extract of *Acacia catechu*. MDA, malondialdehyde; GSH, reduced glutathione; CAT, catalase; SOD, superoxide dismutase; GST, glutathione *S*-transferase; GR, glutathione reductase; GPx, glutathione peroxidase. Data represent the means ± SEM in each group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle treated and DMBA/TPA or DMBA/TPA/AQCE. Comparisons were made between control (Group 1) and DMBA/TPA (Group 2) treated mice and between DMBA/TPA (Group 2) and DMBA/TPA/AQCE (Group 3) treated mice.

increase ($P < 0.05$) in the GSH levels in the tumours of animals treated with DMBA/TPA/AQCE in comparison with the GSH level in the tumours of animals treated with DMBA/TPA. In the liver, a decrease of 74.66% in DMBA/TPA treated animals ($P < 0.001$) and a decrease of 38.83% in DMBA/TPA/AQCE treated animals ($P < 0.001$) in GSH levels was observed in comparison with vehicle treated controls. In general, an increase of 141.48% ($P < 0.001$) in the level of GSH was observed in the livers of animals treated with DMBA/TPA/AQCE in comparison with animals treated with DMBA/TPA (Table 6).

SOD activity

A decrease of 60.83% ($P < 0.001$) in SOD activity was observed in the tumours of DMBA/TPA treated animals, whereas animals treated with DMBA/TPA/AQCE exhibited a 31.46% decrease ($P < 0.01$) compared with controls. There was an average 75% increase ($P < 0.001$) in SOD activity in the tumours of animals treated with DMBA/TPA/AQCE compared with the level in DMBA/TPA treated animals (Table 5). On application of DMBA/TPA, the SOD activity in the liver of these animals showed a 58.35% decrease ($P < 0.01$), whereas a 20.51% decrease ($P < 0.01$) was observed in DMBA/TPA/AQCE treated animals compared with controls. However, the SOD activity in the liver of animals treated with DMBA/TPA/AQCE showed a 90.85% increase ($P < 0.01$) compared with the SOD activity in the animals treated with DMBA/TPA (Table 6).

CAT activity

Compared with vehicle treated control animals, the CAT activity in tumours of animals treated with DMBA/TPA exhibited a 68.97% decrease ($P < 0.001$), and a 47.43% decrease ($P < 0.001$) in DMBA/TPA/AQCE treated animals. In general, there was a 64.65% increase ($P < 0.01$) in CAT activity in the tumours of animals treated with DMBA/TPA/AQCE compared with the activity in the DMBA/TPA treated animals (Table 5). Compared with vehicle treated control animals, the activity of CAT in the livers of animals treated with DMBA/TPA showed a 32.78% decrease ($P < 0.01$) and a 5.38% decrease ($P < 0.05$) in DMBA/TPA/AQCE treated animals. In general, a 40.77% ($P < 0.05$) increase was observed in animals treated with DMBA/TPA/AQCE compared with animals treated with DMBA/TPA (Table 6).

GST activity

Compared with the vehicle treated animals (Group 1) the GST activity in tumourous skin of animals treated with DMBA/TPA (Group 2) showed a decrease of 43.03% ($P < 0.01$), and a 22.23% decrease ($P < 0.01$) in DMBA/TPA/AQCE treated

animals. In general, there was a 36.51% increase ($P < 0.01$) in GST activity in the tumours of animals treated with DMBA/TPA/AQCE compared with the activity in the DMBA/TPA treated animals (Table 5). In comparison with vehicle treated control animals, the GST activity in the livers of animals treated with DMBA/TPA showed a 55.9% decrease ($P < 0.001$) and a 31.54% decrease ($P < 0.01$) in DMBA/TPA/AQCE treated animals. In general, a 55.23% ($P < 0.01$) increase was observed in the animals treated with DMBA/TPA/AQCE compared with the animals treated with DMBA/TPA (Table 6).

GR activity

The GR activity in the skin of animals treated with DMBA/TPA increased by 60.86% ($P < 0.001$), whereas animals treated with DMBA/TPA/AQCE exhibited only a 32.60% ($P < 0.001$) increase compared with vehicle treated control animals. In general, a 17.56% decrease ($P < 0.001$) in the activity of GR was observed in the tumours of animals treated with DMBA/TPA/AQCE in comparison with the GR activity in the tumours of DMBA/TPA treated animals (Table 5). The GR activity increased in the liver by 85.93% ($P < 0.001$) in animals treated with DMBA/TPA, whereas, animals treated with DMBA/TPA/AQCE exhibited a 39.06% increase ($P < 0.01$) in GR activity in the liver in comparison with the vehicle treated control animals. In general, a 25.21% decrease ($P < 0.01$) in the liver GR activity of DMBA/TPA/AQCE treated animals was observed compared with the level in DMBA/TPA treated animals (Table 6).

GPx activity

The GPx activity in the skin of animals treated with DMBA/TPA increased by 88.49% ($P < 0.01$), whereas animals treated with DMBA/TPA/AQCE exhibited only a 42.64% ($P < 0.01$) increase in comparison with vehicle treated control animals. In general, a 24.41% decrease ($P < 0.01$) in GPx activity was observed in the tumours of animals treated with DMBA/TPA/AQCE in comparison with the GPx activity in the tumours of DMBA/TPA treated animals (Table 5). The GPx activity increased in the liver by 88.63% ($P < 0.01$) in animals treated with DMBA/TPA, whereas animals treated with DMBA/TPA/AQCE exhibited a 45.45% increase ($P < 0.01$) in GPx activity in the liver in comparison with the vehicle treated control animals. In general, a 22.89% decrease ($P < 0.01$) in GPx activity in the liver of DMBA/TPA/AQCE treated animals was observed compared with the level in DMBA/TPA treated animals (Table 6).

Discussion

Skin carcinogenesis, the most common of all cancers, has been increasing in recent years worldwide. Skin is the most common site of malignancy and represents 55% of all human cancers, with tremendous impact on health and morbidity. In recent years, much interest has been focused in the identification of natural products that are capable of reducing the tumourigenicity of the environmental carcinogens. In the present study, the cytotoxic effect of AQCE against A431 cells was accessed and further chemopreventive potential of standardized AQCE was screened by monitoring the percentage of tumour bearing animals, tumour volume and burden, as well as by analysing the status of detoxification enzymes, lipid peroxidation and antioxidants in DMBA/TPA treated animals.

A reduction in cell growth and induction of cell death are two major ways to inhibit tumour growth. The cytotoxicity assays employed revealed similar profiles, with the SRB, LDH and MTT assays being the most widely used cytotoxicity assays showing statistically significant IC50 values. In this study, it was observed that the AQCE induces a marked concentration and time-dependent inhibition of A431 cell proliferation, with an IC50 value of 78.56 ± 0.10 μg/ml after 48 h incubation. The results obtained from the three cytotoxicity assays were not in close agreement. This observation can be explained by the nature of each assay. The LDH leakage assay is based on the release of the enzyme into the culture medium after cell membrane damage, whereas the MTT assay is mainly based on the enzymatic conversion of MTT in the mitochondria. The SRB assay is a colorimetric assay measuring the uptake of the dye by metabolically active fixed cells. The chemopreventive study showed a significant increase in tumour latency by administration of AQCE in Balb/c mice initiated by DMBA and promoted by TPA. This may be due to the delay in the promotion phase of carcinogenesis. There was a decrease in the mean tumour burden (84.40%) by the end of the experiment (20 weeks) and a significant reduction in the number of tumours formed per mouse was observed (55.66% until the end of Week 20). A significant decrease in the final bodyweight of DMBA/TPA mice (Group 2) when compared with initial bodyweight indicated a decrease in growth probably owing to tumour burden. However, the administration of AQCE (Group 3) showed normal growth, similar to control mice. The histopathological investigations showed a normal histological pattern in the skin of the control group (Group I). In DMBA/TPA treated mice, all the tumours confirmed to be papillomas showed necrotic keratinised squamous pearls, suggesting invasive squamous cell carcinoma. On the other hand, an intact basal cell layer and dysplastic lesions characterized benign papillomas in DMBA/TPA/AQCE treated animals. This direct evidence shows that AQCE administration inhibits carcinoma formation and conversion of papillomas into frank squamous cell carcinoma.

The liver, the major metabolic organ, performs an important role in the detoxification process and therefore analysing the status of detoxification agents helps to identify the chemopreventive efficacy of the test compound. Skin cancer chemopreventive agents protected against tumour formation by activating multiple biochemical mechanisms including phase-II detoxification enzyme induction and the antioxidant defence mechanism.[27,28] Phase-II detoxification enzymes play a major role in increasing the polarity and assisting the excretion of xenobiotic agents. They detoxify carcinogens either by destroying their reactive centres or by conjugating them with endogenous ligand, facilitating their excretion. A large number of experimental studies report that chemopreventive agents convert DNA damaging entities through the induction of detoxication agents such as GST.[29]

The activity of GST and GSH content were decreased in DMBA/TPA mice compared with control mice. Oral administration of AQCE increased the activity of phase-II detoxification agents in DMBA/TPA treated mice, which indicates

that AQCE stimulated the activity of phase-II detoxification enzymes to facilitate the excretion of the active metabolite of DMBA, dihydrodiol epoxide. Overproduction of reactive oxygen species in the cell cause DNA damage, thereby contributing to carcinogenesis. Enzymatic and non-enzymatic antioxidants form the first line of defence against reactive oxygen species mediated lipid peroxidation. The low levels of antioxidant enzymes GST, SOD and CAT in DMBA/TPA treated mice indicate poor antioxidant status. Oberley and Oberley[30] have also reported decreased activity of SOD and CAT in squamous cell carcinomas. The increase in antioxidant enzymes by AQCE suggests that it inhibits the process of oxidative stress induced carcinogenesis. Several reports suggest that GSH is a more efficient antioxidant agent than SOD or CAT.[31] GSH alters the profiles of lipoxygenase and cyclooxygenase,[32,33] which are involved in tumourigenesis. Oberley and Oberley[30] reported decreased SOD and CAT activity in papillomas and squamous cell carcinoma, leading to a pro-oxidant state of cells, facilitating tumourigenesis. However, GSH has been found to be highly variable and contradictory, depending on the cell type, nature of the carcinogen and its modulatory pathways.[30,34,35] An increase in the level of GSH by the chemopreventive action of flavonoids in mouse skin has also been reported.[36] It is thus the combined effect of modulating antioxidant enzymes that may lead to a favourable shift in the intracellular oxidation/reduction balance, resulting in downregulation of lipid peroxidation. They are also known to inhibit the in-vivo metabolism of DMBA and other procarcinogens to metabolites capable of binding to DNA.[37]

During oxidative stress, malonaldehyde and other aldehydes are formed in the biological system as a result of lipid peroxidation. This can cause chaotic cross-linkage between proteins and nucleic acids, resulting in alteration in replication and transcription,[38] leading to tumour promotion. Elevated levels of MDA in skin tumours as well as in livers of animals treated with DMBA/TPA suggests oxidative stress in DMBA/TPA-induced mouse skin carcinogenesis. A significant decrease in MDA levels by AQCE treatment indicates its role in reducing oxidative stress, thus indicating its protective potential against skin carcinogenesis. This protective effect of AQCE as indicated by reduced lipid peroxides could be due to an increase in GSH and the antioxidant enzymes CAT and SOD. The decrease in lipid peroxides may also explain the reduced invasiveness of the tumour as indicated by the histopathology of tumours of DMBA/TPA/AQCE treated animals.

Conclusions

It seems at this point of investigation that the chemopreventive effect of AQCE may be at least in part due to its antioxidative properties. Further investigations are in progress in our laboratory to identify the active principles involved in its anticancer and antioxidant activity.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Sporn MB. The war on cancer. *Lancet* 1996; 347: 1377–1381.
2. Sporn MB *et al*. Chemoprevention of cancer. *Carcinogenesis* 2001; 21: 525–530.
3. Mradhu G *et al*. Therapeutic utilization of secretory products of some Indian medicinal plants – a review. *Indian J Tradit Knowl* 2006; 5: 569–575.
4. Chandra PK *et al*. Developing the medicinal plants sector in northern India: challenges and opportunities. *J Ethnobiol Ethnomedicine* 2006; 2: 32.
5. Ray D *et al*. Antipyretic, antidiarrhoeal, hypoglycaemic and hepatoprotective activities of ethyl acetate extract of *Acacia catechu*. *Indian J Pharmacol* 2006; 38: 408–413.
6. Nongyao S, Kitja S. The effects of extracts from anti-diarrheic Thai medicinal plants on the in vitro growth of the intestinal protozoa parasite: *Blastocystis hominis*. *J Ethnopharmacol* 2005; 98: 67–72.
7. Supayang V *et al*. Effective medicinal plants against enterohaemorrhagic *Escherichia coli* O157:H7. *J Ethnopharmacol* 2004; 94: 49–54.
8. Naik GH *et al*. Comparative antioxidant activity of individual herbal components used in Ayurvedic medicine. *Phytochemistry* 2003; 63: 97–104.
9. Fadeyi MQ *et al*. Epidermal and phytochemical studies in the genus *Boerhavia* (Nyctanginaceae) in Nigeria. *Int J Crude Drug Res* 1989; 29: 178–184.
10. Kapoor LD *et al*. Survey of Indian medicinal plants for saponins, alkaloids and flavonoids. *Lloydia* 1969; 32: 297–302.
11. Odebiyi A, Sofowora AE. Phytochemical screening of Nigerian medicinal plants. Part III. *Lloydia* 1990; 41: 234–246.
12. Rizk AM, Bashir A. Chemical survey of sixty plants. *Fitoterapia* 1980; 53: 35–44.
13. Jame KDN *et al*. Cytotoxic potential of the phytochemical genistein isoflavone and certain environmental chemical compound on testicular cells. *Biol Cell* 1999; 91: 515–523.
14. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 1986; 89: 271–277.
15. Vanicha V, Kanyawim K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature* 2006; 1: 1112–1116.
16. Skehan P *et al*. New colorimetric cytotoxicity assay for anti cancer drug screening. *J Natl Cancer Inst* 2003; 82: 1107–1112.
17. Bashar S *et al*. Evaluation of medicinal plant hepatotoxicity in co-cultures of hepatocytes and monocytes. *Evid Based Complement Alternat* 2006; 3: 93–98.
18. Subapriya R, Nagini S. Ethanolic neem leaf extract protects against N-methyl-N-nitro-nitrosoguanidine induced gastric carcinogenesis. *Asian Pac J Cancer Prev* 2003; 4: 215–223.
19. Moron MS *et al*. Levels of glutathione, glutathione reductase and glutathione *S*-transferase activities in rat lung and liver. *Biochim Biophys Acta* 1979; 582: 67–71.
20. Ohkawa H *et al*. Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351–358.
21. Habig WH *et al*. Glutathione *S*-transferase, the first enzymatic step in marcapturic acid formation. *J Biol Chem* 1974; 249: 7130–7139.
22. Abie HE. Catalase. In: Bermeyer HU, ed. *Methods of Enzymatic Analysis*. New York: Academic Press, 1974: 276–286.

23. Kono Y. Generation of superoxide radical during auto-oxidation of hydroxylamine and assay for superoxide dismutase. *Arch Biochem Biophys* 1978; 186: 189–195.
24. Carlberg I, Mannervik B. Purification and characterization of the flavo enzyme glutathione reductase from rat liver. *J Biol Chem* 1975; 250: 5475–5480.
25. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967; 70: 158–169.
26. Burnett BP *et al*. A medicinal extract of *Scutellaria baicalensis* and *Acacia catechu* acts as a dual inhibitors of cyclooxygenase and 5-lipoxygenase to reduce inflammation. *J Med Food* 2007; 10: 442–451.
27. Keeney S *et al*. Attitudes, knowledge and behaviours with regard to skin cancer: a literature review. *Eur J Oncol Nurs* 2009; 13: 29–35.
28. Meijerman I *et al*. Combined action and regulation of phase II enzymes and multidrug resistance proteins in multidrug resistance in cancer. *Cancer Treat Rev* 2008; 34: 505–520.
29. Choi SC *et al*. Prognostic potential of glutathione S-transferase M1 and T1 null genotypes for gastric cancer progression. *Cancer Lett* 2003; 195: 169–175.
30. Oberley TD, Oberley LW. Oxygen radicals and cancer. In: Yu BP, ed. *Free Radicals in Aging*. Boca Raton, FL: CRC Press, 1993: 247–267.
31. Michiels C *et al*. Importance of Se-glutathione peroxidase, catalase and Cu/Zn SOD for cell survival against oxidative stress. *Free Radic Biol Med* 1994; 17: 235–248.
32. Capdevila JH *et al*. The catalytic outcomes of the constitutive and the mitogen inducible isoforms of prostaglandin H synthetase are markedly affected by glutathione and glutathione peroxidase(s). *Biochemistry* 1995; 34: 3325–3337.
33. Bryant RW *et al*. Role of glutathione peroxidase and monophosphate shunt in the platelet lipoxygenase pathway. *J Biol Chem* 1982; 257: 14937–14943.
34. Oberley LW, Oberley TD. Role of antioxidants in cell immortalization and transformation. *Mol Biochem* 1988; 84: 147–153.
35. Machlemater D *et al*. Active oxygen induced DNA strand breakage and poly ADP ribosylation in promotable and nonpromotable JB6 mouse epidermal cells. *Carcinogenesis* 1988; 9: 239–245.
36. Elangovan V *et al*. Studies on the chemopreventive potential of some naturally occurring bioflavonoids in 7,12-dimethylbenz(a)anthracene induced carcinogenesis in mouse skin. *J Clin Biochem Nutr* 1994; 17: 153–160.
37. Liu JZ *et al*. Inhibition of 7, 12-dimethylbenz (a) anthracene-induced mammary tumors and DNA adducts by dietary selenite. *Cancer Res* 1991; 51: 4613–4617.
38. Perchellet JP, Perchellet M. Antioxidant and multistage carcinogenesis in mouse skin. *Free Radic Biol Med* 1989; 7: 377–408.