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Original Article

Isolation and characterization of flavanols from *Anthocephalus cadamba* and evaluation of their antioxidant, antigenotoxic, cytotoxic and COX-2 inhibitory activities

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ABSTRACT

In search of lead molecules for use in disease prevention and as food additive from natural sources, two flavanols were isolated from leaves of *Anthocephalus cadamba* (Roxb.) Miq., Rubiaceae. Their structures were established as 6-hydroxycoumarin-(4″→8)-(−)-epicatechin and 6-hydroxycoumarin-(4″→8)-(−)-epicatechin-(4→6‴)-(−)-epicatechin on the basis of spectroscopic data. Both the compounds exhibited potent antioxidant and antigenotoxic activity. 6-Hydroxycoumarin-(4″→8)-(−)-epicatechin scavenged DPPH, $ABTS^{+\cdot}$ and superoxide anion radicals with IC_{50} values of 6.09 μg/ml, 5.95 μg/ml and 42.70 μg/ml respectively whereas the IC_{50} values for 6-hydroxycoumarin-(4″→8)-(−)-epicatechin-(4→6‴)-(−)-epicatechin were 6.62 μg/ml for DPPH free radicals, 6.93 μg/ml for ABTS radical cations and 49.08 μg/ml for superoxide anion radicals. Both the compounds also exhibited potent reducing potential in reducing power assay and protected the plasmid DNA (pBR322) against the attack of hydroxyl radicals generated by Fenton's reagent in DNA protection assay. In SOS chromotest, 6-hydroxycoumarin-(4″→8)-(−)-epicatechin decreased the induction factor induced by 4NQO (20 μg/ml) and aflatoxin B1 (20 μg/ml) by 31.78% and 65.04% respectively at a concentration of 1000 μg/ml. On the other hand, 6-hydroxycoumarin-(4″→8)-(−)-epicatechin-(4→6‴)-(−)-epicatechin decreased the genotoxicity of these mutagens by 37.11% and 47.05% respectively. It also showed cytotoxicity in COLO-205 cancer cell line with GI_{50} of 435.71 μg/ml. Both the compounds showed moderate cyclooxygenase-2 inhibitory activity.

Introduction

Cancer is a major public health problem in all parts of the world. Diverse molecular, biochemical and cellular mechanisms at each stage of carcinogenesis are accountable for altering normal cell cycle process and converting a normal cell to a cancerous one (Seifried et al., 2007). It has been observed that about 20% or more of cancer cases could be prevented by increased intake of fruits and vegetables in daily diet (Ruhul Amin et al., 2009). Natural products/phytochemicals are being explored for their chemopreventive properties due to their non-toxic nature, protective effects against oxidants and their recognition as dietary additives (Suh et al., 2009; Lin et al., 2011; Liu et al., 2011; Wang et al., 2011; Kundu et al., 2014; Bohn et al., 2014). Oxidative stress is the major determinant in the development of diverse diseases (Halliwell, 1997; Flora, 2007; Halliwell and Gutteridge, 2007; Ziech et al., 2010; Ayala-Pena, 2013). Plant derived constituents provide protection from ROS-induced DNA damage and consequently from carcinogenesis (Lamson et al., 2010). Numerous reports have shown the capability of phytoconstituents to provide protection against free radical induced ailments (Sahin et al., 2010; Ungvari et al., 2010; Negi et al., 2011; Scapagnini et al., 2011; Xiong et al., 2012; Ziech et al., 2012; Carmona-Ramirez et al., 2013). Phytochemicals isolated from different parts of the plants belonging to diverse classes of plant secondary metabolites are accountable for antioxidant properties of medicinal plants (Chung et al., 1998; Pietta, 2000). Toxic effects of antioxidants of synthetic origin have limited their utilization in food products (Ito et al., 1986; Peters et al., 1996; Li et al., 2002). Natural plant products are frequently reported as efficient chemopreventive agents (Surh and Ferguson, 2003). Immense research carried out in plant sciences has lead to the identification

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of various plants used in ancient times for their medicinal potential.

Anthocephalus cadamba (Roxb.) Miq., Rubiaceae, is an Ayurvedic medicinal plant, used in treating various ailments. It is used as a folk medicine in the treatment of fever and anemia, as antidiuretic and for improvement of semen quality. Earlier, in a study from the same laboratory, we have reported that among all fractions, EAAC fraction of *A. cadamba* leaves exhibited highest potential to counter oxidative stress in various *in vitro* antioxidant assays (Chandel et al., 2012). Therefore, the present study was undertaken toward an effort to isolate the active compounds responsible for the potential antioxidant activity of EAAC fraction and to evaluate the isolated molecules for their antioxidant/antigenotoxic/cytotoxic properties.

Materials and methods

Bacterial strain/cell lines and chemicals

Escherichia coli PQ37 strain was purchased from Institut Pasteur, France. HeLa and COLO-205 cancer cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, L-ascorbic acid, NADH (nicotinamide adenine dinucleotide), PMS (phenazine methosulphate), NBT (nitroblue tetrazolium chloride), *ortho*-nitrophenyl β-D-galactopyranoside (ONPG), *para*-nitrophenylphosphate (PNPP), fetal bovine serum (FBS), DMEM culture medium, RPMI culture medium, MTT, dimethyl sulfoxide (DMSO), penicillin, trypsin, antibiotic/antimycotic solution, HEPES, $NaHCO_3$, streptomycin were obtained from HiMedia Pvt. Limited Mumbai, India. Potassium persulfate, ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] and rutin from Sigma (St. Louis, MO, USA). Plasmid pBR322 was purchased from Genei Pvt. Ltd., Banglore. All other reagents used were of analytical grade (AR).

Collection of plant material

The plant material used (leaves of *Anthocephalus cadamba* (Roxb.) Miq., Rubiaceae) was procured during July 2010 from campus of Guru Nanak Dev University (G.N.D.U.), Amritsar, Punjab (India). The specimen was identified and kept at Herbarium, Department of Botanical and Environmental Sciences, G.N.D.U. with Voucher specimen no. 6557/2011.

Column chromatography of EAAC fraction

EAAC fraction (15 g) was prepared as mentioned in the fractionation procedure (Fig. 1) and dissolved in 10 ml of MeOH, mixed with silica gel and the slurry was made. The column was eluted using a gradient of hexane/EtOAc (100/0), (98/2), (95/5), (85/15), (80/20), (75/25), (70/30), (60/40), (50/50), (40/60), (30/70), (20/80), (10/90), (0/100) and finally the column was eluted with MeOH. Three fractions were collected when eluting with hexane/EtOAc ((10/90) *viz.* EALAC1, EALAC2 and EALAC3. A gradient of hexane/EtOAc (100/0), (75/25), (70/30), (65/35), (60/40), (55/45), (50/50), (40/60), (20/80), (0/100) was used to further fractionate EALAC2 (500 mg) and the column was eluted with MeOH. Compound **1** (20 mg) was obtained from fractions eluting in (55:45) *n*-hexane/EtOAc.

EALAC3 (7.91 g) was dissolved in EtOAc and washed with H_2O (3 × 300 ml). The EtOAc layer was dried over sodium sulfate and concentrated to obtain EALAC3-S fraction. A gradient of $CHCl_3$/MeOH (100/0), (95/5), (92/8), (90/10), (85/15), (80/20), (75/25), (70/30), (60/40), (50/50), (30/70), (0/100) was used to column chromatograph EALAC3-S fraction and finally elution was done with MeOH. Thin layer chromatography of fractions collected in $CHCl_3$/MeOH (90/10) gave single spot and the fractions were concentrated and lyophilized to obtain dark brown colored compound **2** (50 mg). Compound **1** was again obtained from precipitates obtained at gradient $CHCl_3$/MeOH (95/5) as mentioned in Fig. 1.

Antioxidant activity

DPPH-radical scavenging assay

Scavenging of DPPH radicals was assayed using the protocol of Blois (1958) with minor modifications.

ABTS radical scavenging assay

The spectrophotometric analysis of $ABTS^{+\bullet}$ scavenging activity was determined according to the protocol given by Re et al. (1999) with slight modifications.

Reducing power assay

Reducing potential of both the compounds was determined using the method of Oyaizu (1986).

Superoxide anion radical scavenging assay

The measurement of superoxide anion scavenging activity of the isolated compounds was performed according to the method of Nishikimi et al. (1972) with slight modifications.

Rutin was used as standard antioxidant compound in DPPH, $ABTS^{\cdot+}$, reducing power and superoxide anion radical scavenging assays.

DNA protection assay

To measure the hydroxyl radical scavenging effect of the isolated compounds, DNA nicking experiment was performed according to the protocol of Lee et al. (2002).

Antigenotoxic activity

SOS chromotest

The SOS chromotest is an SOS transcriptional-fusion-based assay, which is able to estimate primary DNA damage produced by chemicals and physical agents by measuring the expression of a reporter gene (β-galactosidase) that becomes colored in the presence of a substrate. It was carried out with the method of Quillardet and Hofnung (1985). Exponential-phase culture of *E. coli* PQ37 was grown at 37 °C in L medium (1% bactotryptone, 0.5% yeast extract and 1% NaCl) supplemented with 20 μg/ml ampicillin. Overnight culture (1 ml) was diluted in 9 ml of fresh L medium for the assay without metabolic activation or 9 ml of S9 mix for assay with metabolic activation. Aliquots (600 μl) of above mixture containing 20 μl of genotoxicant [4NQO/AFB1 (20 μg/ml)] and tested fractions (compounds **1** and **2**) of different concentrations (10–1000 μg/ml) were distributed into glass test tubes. Positive control was prepared by exposure of bacteria to 4NQO/AFB1. After incubation of 2 h at 37 °C, 300 μl samples were used for assay of β-galactosidase and alkaline phosphatase activities respectively. The activity of the constitutive enzyme alkaline phosphatase was used as a measure of protein synthesis and toxicity. In order to determine the β-galactosidase activity, 2.7 ml of B-buffer (adjusted to pH 7.5) was added and after 10 min, 600 μl of 0.4% 4-nitrophenyl-β-galactopyranoside (ONPG) solution was added to each of the test tubes of one set. To determine the constitutive alkaline phosphatase activity, P-buffer (adjusted to pH 8.8) was added and after 10 min, 600 μl of 0.4% 4-nitrophenyl phosphate (PNPP) solution was added to another set of tubes. All mixtures were incubated at 37 °C and observed for the color development. After 30 min, the conversion of ONPG was stopped with 2 ml of 1 M sodium carbonate and that of PNPP with 1 ml of 2.5 M HCl and after 5 min added 1 ml of 2 M tris

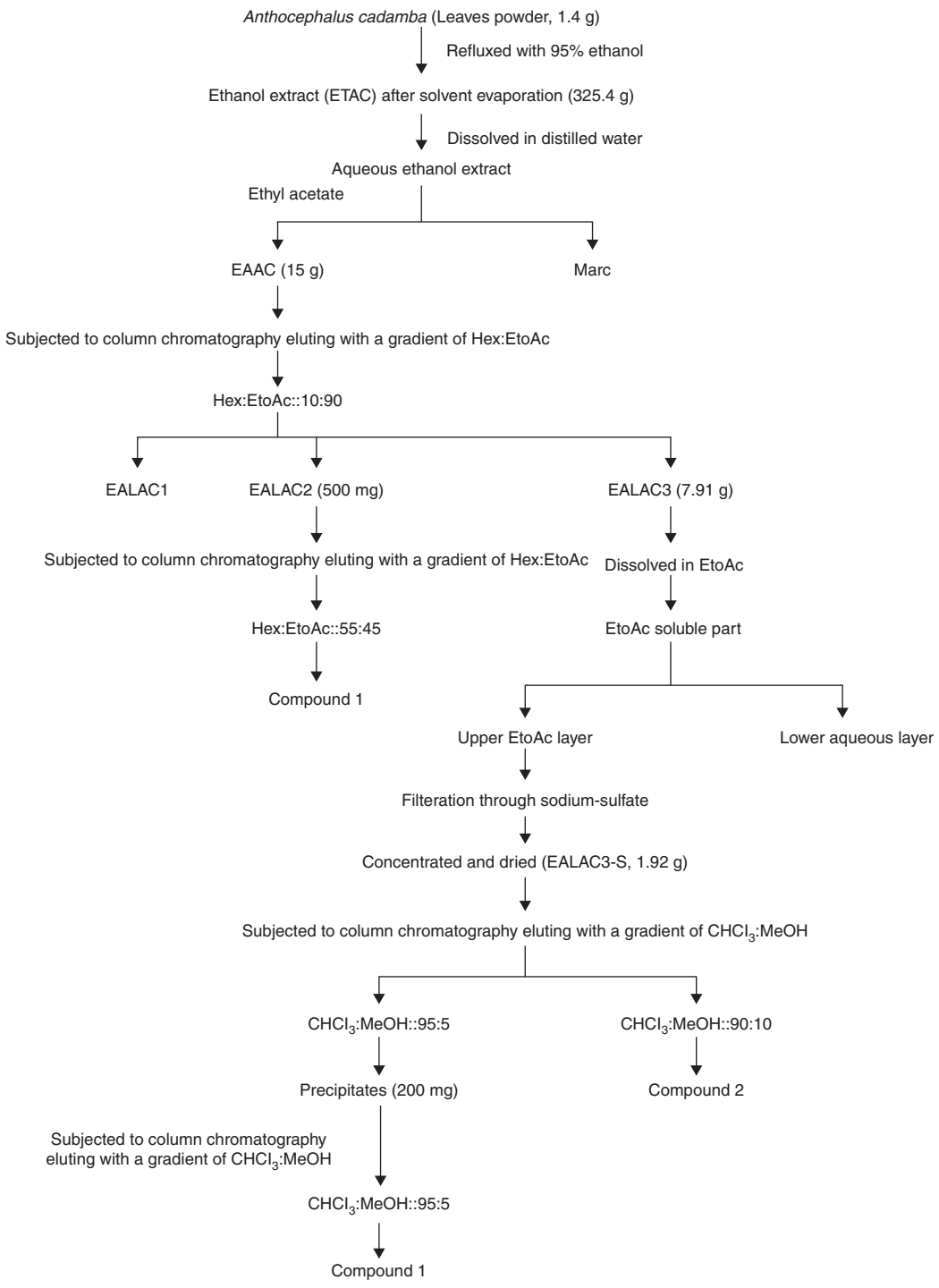


Fig. 1. Isolation of compounds **1** and **2** from leaves of *Anthocephalus cadamba*.

(hydroxymethyl)amino-methane. The absorption was measured at 420 nm using a reference solution in which culture is replaced by L medium.

The enzyme activities were calculated according to the simplified method:

$$\text{Enzyme units} \quad (\text{U}) = \frac{A_{420} \times 1000}{t}$$

A_{420}: optical density at 420 nm; t: substrate conversion time in minutes.

$$\text{Induction factor} \quad (\text{IF}) = \frac{\text{Rc}}{\text{Ro}}$$

Rc: β-galactosidase activity/alkaline phosphatase activity determined for the test compound at concentration c,

Ro: β-galactosidase activity/alkaline phosphatase activity in the absence of the test compound.

Anti-genotoxicity was expressed as percentage inhibition of genotoxicity according to the formula:

$$\text{Inhibition} \quad (\%) = 100 - \frac{\text{IF}_1 - \text{IF}_0}{\text{IF}_2 - \text{IF}_0} \times 100$$

where:

IF_1 is the induction factor of the test compound

IF_2 is the induction factor of positive control (4NQO and aflatoxin B1)

IF_0 the induction factor of the blank (without any test compound).

Cytotoxicity

MTT assay

The cytotoxicity of compounds **1** and **2** on Hela and COLO-205 cancer cell lines was assessed by MTT assay. Cell plating (1×10^4/well) was done in 96-well plates. After 24 h incubation, cells were incubated with different concentrations of the test sample in DMSO (0.1%) for 24 h. Addition of 10 μl of MTT (5 mg/ml, phosphate-buffered saline solution) was done followed by 2 h incubation. The medium was discarded and DMSO was used to dissolve the crystals of reduced MTT formed. Optical density was noticed at 570 nm. The % of cytotoxicity was determined using the formula give below:

$$\%\,\text{cytotoxicity} = \frac{A0 - A1}{A0} \times 100$$

where,

A_0 = absorbance of control

A_1 = absorbance of test sample

In vitro *COX-2 inhibitory activity*

Inhibition of COX-2 activity of isolated compounds from EAAC fraction from leaves of *A. cadamba* was assessed with the help of 'COX (ovine/human) inhibitor screening assay' kit (Item No. 560131, Cayman Chemicals Company, USA).

Statistical analysis

The results were presented as the mean ± standard error. Regression analysis was carried out by best fit method and IC_{50} values were calculated using regression equation. The data were analyzed for statistical significance using analysis of variance (one-way ANOVA). The difference among average values was compared by honestly significant difference (HSD) using Tukey's test. The significance was checked at $^*p \leq 0.05$.

Results and discussion

*Analysis of compounds **1** and **2***

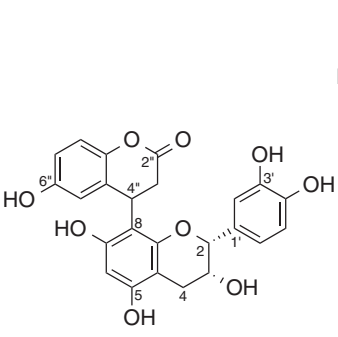
*Compound **1***

HRMS of compound **1** displayed a molecular ion peak at *m/z* 452.7750 corresponding to the molecular formula $C_{24}H_{20}O_9$ (see supplementary data associated with this article). Total twenty four carbon signals were observed in ^{13}C NMR spectra of compound **1** including ten methines, two methylenes and twelve quaternary as evident from DEPT spectra. Signal for methylene at δ_C 28.2 (C-4) correlating with δ_H 2.86 (1H, m) and δ_H 2.94 (1H, m), in HMQC spectrum was found to be adjacent to two oxygenated methines at δ_H 4.20 (1H, m), δ_C 66.0 (C-3) and δ_H 4.82 (1H, overlapped), δ_C 79.1 (C-2) by HMBC analysis, hence, suggested the presence of flavan-3-ol type of unit in compound **1**. Three aromatic signal at [δ_H 6.84 (1H, s); δ_C 114.9 (C-2′)], [δ_H 6.72 (1H, m), δ_C 115.5 (C-5′)] and [δ_H 6.69 (1H, m), δ_C 118.4 (C-6′)] showed long range correlation with quaternary aromatic carbons at δ_C 130.7 (C-1′), δ_C 144.9 (C-3′) and δ_C 145.3 (C-4′) in HMBC spectra of compound **1**. Other oxygenated aromatic carbons C-5, C-7, C-9, C3′ and C4′ were observed at δ 156.2, 152.5, 151.0, 144.9 and 145.3 respectively. On the basis of these NMR chemical shifts and comparison with literature values (Agrawal and Bansal, 1989) partial skeleton of compound **1** appeared to be (−)-epicatechin.

In ^1H NMR spectrum, only one singlet for H-6 was observed instead of *meta*-coupled protons for H-6 and H-8 in case of (−)-epicatechin that indicated the presence of substitution at C-8. ^{13}C NMR spectrum of compound **1** showed additional signals for ester type carbonyl at δ_C 169.8, methylene at [δ_H 2.83–2.88 (2H, m), δ_C 37.2 (C-3″)], aliphatic methine at [δ_H 4.45 (12H, m), δ_C 34.3 (C-3″)], aromatic methines [δ_H 6.78 (1H, m), δ_C 114.3 (C-5″)], [δ_H 6.63 (1H, m), δ_C 114.0 (C-7″)], [δ_H 6.99 (1H, m), δ_C 118.3 (C-8″)] and three quaternary carbons at δ_C 152.5 (C-6″), 144.1 (C-9″) and 134.3 (C-10″). The analysis of these NMR values suggested 6-hydroxycoumarin skeleton. The substitution at C-8 was confirmed by ^{13}C NMR spectrum which revealed downfield quaternary carbon for C-8 at δ_C 105.1. This quaternary carbon at δ 105.1 (C-8 of (−)epicatechin) showed long range correlations (HMBC) with methine at δ 34.3 (C-4″ of 6-hydroxycoumarin) suggested that both moieties were C-C linked which was further confirmed from spectral data. Thus, on the basis of NMR spectral data (see supplementary data associated with this article) the structure of compound **1** was elucidated as 6-hydroxycoumarin-(4″→8)-(−)-epicatechin. The structure is tentatively assigned as for coumarin unit linkage at C-6 instead of C-8 similar NMR values and correlation are expected.

*Compound **2***

HRMS of compound **2** displayed a molecular ion peak at *m/z* 741.8964 (see supplementary data associated with this article) corresponding to the molecular formula $C_{39}H_{32}O_{15}$. ^1H and ^{13}C NMR signals are very similar to the NMR of compound **1** with additional signals corresponding to one more epicatechin moiety. The linkage between two epicatechins units were established by long range correlation between [δ_H 2.97–3.07 (1H, m), δ_C 36.7 (C-4)] C-4 and δ_C 107.3 (C-6‴) in HMBC spectrum (see supplementary data associated with this article). Thus, on the basis of NMR spectral data, 'compound **2**' was characterized as 6-hydroxycoumarin-(4″→8)-(−) epicatechin-(4→6‴)-(−)-epicatechin.



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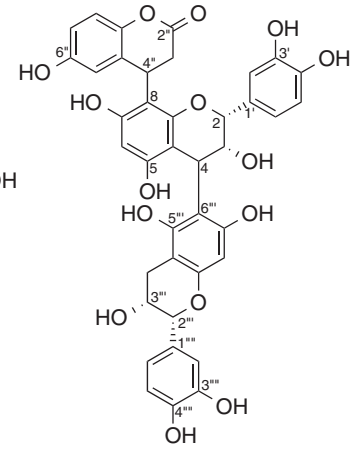

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Antioxidant activity

Compounds **1** and **2** exhibited potent radical scavenging activity in DPPH assay. The compounds **1** and **2** scavenged the DPPH radicals by 77.08% and 76.91% respectively with IC_{50} of 6.09 μg/ml (compound **1**) and 6.62 μg/ml (compound **2**) which was lower than the standard antioxidant compound rutin (IC_{50} 54.05 μg/ml) (Figs. 2 and 3). The compounds also exhibited potent ABTS radical cation scavenging effect. At a concentration of 1 μg/ml the

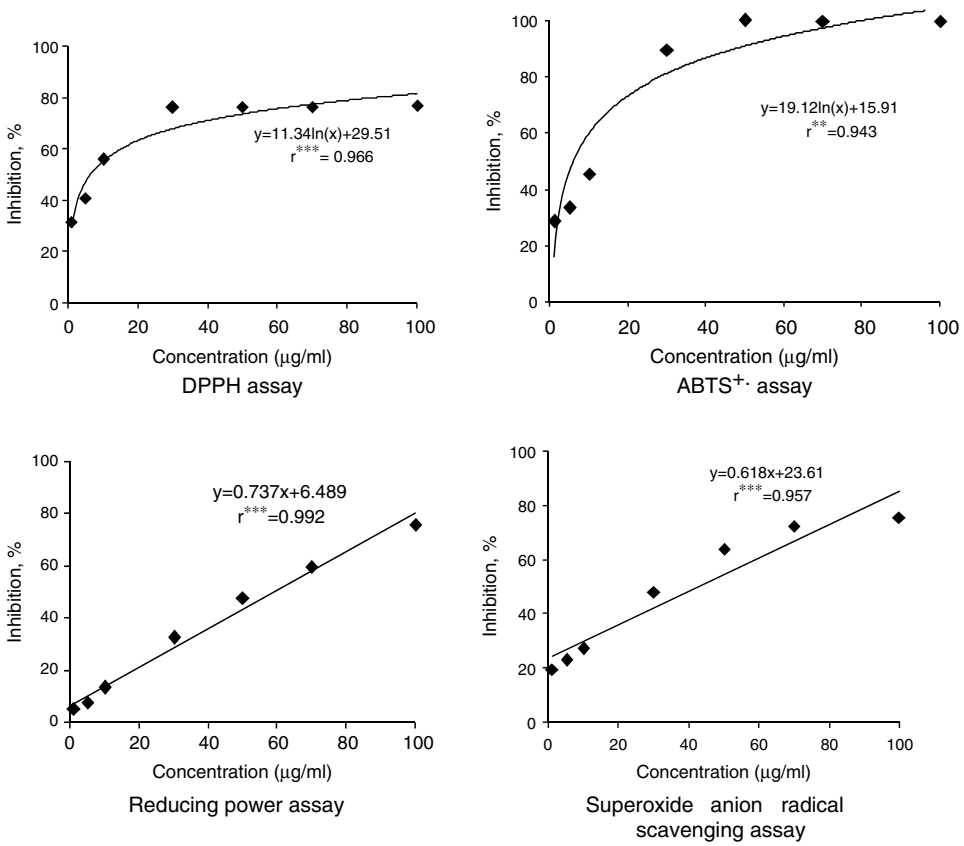

Fig. 2. Antioxidant activity of compound **1** from *Anthocephalus cadamba* leaves in different assays.

scavenging effect exhibited by compounds **1** and **2** was 28.35% and 24.99% which dose dependently increased to 99.76% and 99.59% respectively at the highest tested concentration of 100 μg/ml. The IC_{50} value for compound **1** was calculated as 5.95 μg/ml, whereas compound **2** showed IC_{50} value of 6.93 μg/ml which was lower than the standard antioxidant compound rutin (Figs. 2 and 3). In reducing power assay both the compounds *viz.* **1** and **2** showed potent reduction potential of 76.00% and 55.57% at highest tested concentration (100 μg/ml) respectively. Standard antioxidant compound rutin showed higher IC_{50} (IC_{50} of rutin 160.77 μg/ml) than the isolated compounds (IC_{50} of compound **1**: 70.27 μg/ml; IC_{50} of compound **2**: 85.48 μg/ml with respect to rutin) (Figs. 2 and 3). Both compounds *viz.* compounds **1** and **2** exhibited pronounced superoxide anion radical scavenging with IC_{50} of 42.70 μg/ml and 49.08 μg/ml respectively which was less than that of standard rutin (IC_{50} 58.75 μg/ml) (Figs. 2 and 3). Both the compounds showed the ability to protect the damaged pBR322 plasmid DNA from the attack of hydroxyl radicals generated by Fenton's reaction (Fig. 4).

The higher antioxidant activity of compounds **1** and **2** might be attributed to the presence of more hydroxyl groups present in the A and B ring of the molecules. The number and configuration of hydroxyl groups and the arrangement of functional groups about the nuclear structure may be responsible for the antioxidant capacity of phenolics (Cao et al., 1997; Shekher Pannala et al., 2001).

The results of the present study are in accordance with the several reports showing the potent antioxidant activity of flavonoids (Horvathova et al., 2003; Gulcin et al., 2005; Rahman et al., 2006; Kalpana et al., 2009; Emam et al., 2010; Ho et al., 2012; Jayasinghe et al., 2012; Tatsimo et al., 2012). There is a clear relationship between the antioxidant power and the structural characteristics of flavonoids. The potent antioxidant activity of compounds **1** and **2** might be due to the presence of *O*-dihydroxy groups in the B-ring, the *meta* 5,7-dihydroxy arrangements in the A ring and a -OH group at position 3. Various studies reported the presence of above mentioned structural characteristics as the major determinants of antioxidant activity of flavonoids (Ratty and Das, 1988; Bors et al., 1990; Van Acker et al., 1996; Rice-Evans et al., 1997; Pietta, 2000; Lopez-Velez et al., 2003; Villano et al., 2005; Wolfe and Liu, 2008).

Moreover, flavonoids can terminate radical chain reactions by serving as electron and hydrogen donors and thereby converting free radicals to more stable products (Kelly et al., 2002; Yen and Chen, 1995). The superoxide anions scavenging activity and antioxidation of flavonols (quercetin, rutin, morin), flavones (acacetin, hispidulin) and flavanones (hesperidin, naringin) was studied by Yuting et al. (1990). Rutin was found to be the most potent scavenger followed by quercetin and naringin, while morin and hispidulin were very weak. Cai et al. (1997) attributed the anticarcinogenic effects of different classes of flavonoids *i.e.* flavonol (quercetin), flavones (luteolin), and isoflavone (genistein) to their antioxidant activity. Quercetin and luteolin were found to be potent scavenger of H_2O_2 and superoxide anion radicals and also inhibited the lipid peroxidation efficiently, while genistein showed a moderate effect in radical scavenging and exhibited weak inhibitory effect in lipid peroxidation assay.

Vinson et al. (1995) studied the antioxidant activity of flavonoids and related compounds using an *in vitro* lipoprotein oxidation model and found flavonols in tea to be the most powerful natural antioxidants. Devasagayam et al. (1995) studied the protective effects of flavonols (rutin, myricetin, fisetin), flavanol (+catechin) and flavones (luteolin and apigenin) against singlet molecular oxygen induced single-stranded breaks using plasmid pBR322 DNA. Among the tested compounds myricetin showed highest protective ability and was found more effective than that of other known antioxidants such as lipoate, alphatocopherol and beta-carotene. Gao et al. (1999) examined the free radical scavenging and antioxidant activities of flavones such as baicalein, baicalin, wogonin and

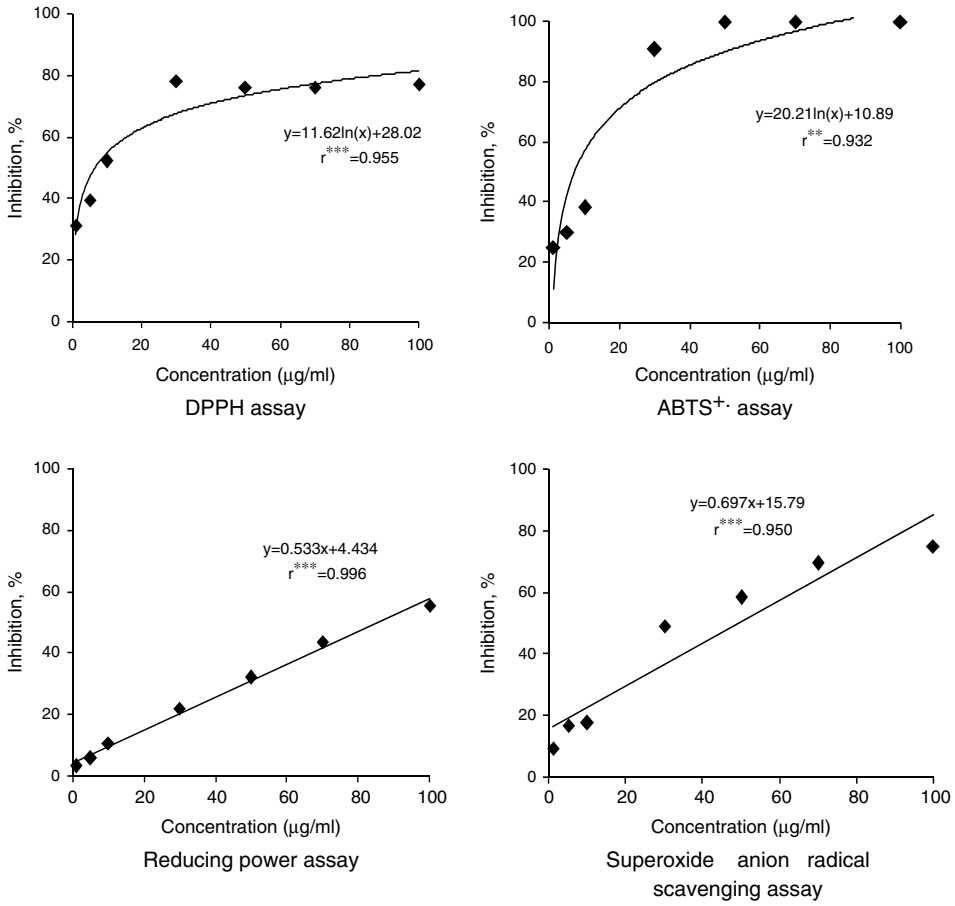

Fig. 3. Antioxidant activity of compound **2** from *Anthocephalus cadamba* leaves in different assays.

wogonoside isolated from radix of *Scutellaria baicalensis*. Dose-dependent scavenging of hydroxyl radicals, DPPH radicals and alkyl radicals was observed by baicalein and baicalin while wogonin and wogonoside showed very weak inhibitory effects on these radicals. Among all the tested compounds baicalein was the most effective antioxidant and its potent antioxidant activity was attributed to the presence of *o*-tri-hydroxyl structure in the A ring. Hirano et al. (2001) evaluated the DPPH• scavenging activity of flavanols (catechin, epicatechin [EC], epigallocatechin [EGC], epicatechin gallate [ECG], epigallocatechin gallate [EGCG]), flavonols (myricetin, quercetin, kaempferol) and flavones (apigenin and luteolin). EGCG was the most potent DPPH radical scavenger, while luteolin was the least active. They have also evaluated the effect of flavonoids on LDL oxidation and the inhibitory effect was in the order of luteolin > ECG > EC > quercetin > catechin > EGCG > EGC > myricetin > kaempferol > apigenin.

Various other workers gave a comparative account of antioxidant activity of flavonoids in different *in vitro* antioxidant assays and attributed the difference in their activity to the presence or absence of substituents on ring A, ring B or ring C (Gao et al., 1999; Pietta, 2000; Mira et al., 2002; Khanduja and Bhardwaj, 2003; Emam et al., 2010).

Antigenotoxic activity

Identification of natural products with specific molecular and cellular targets can provide an effective approach to cancer chemoprevention. Such an approach can be accomplished

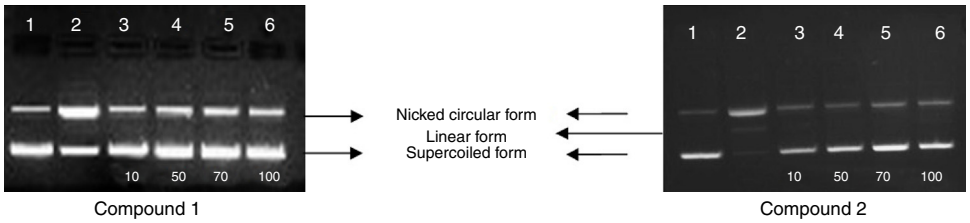

Figure 4. Effect of compounds **1** and **2** from *Anthocephalus cadamba* leaves in plasmid DNA nicking assay.
Lane 1: Negative Control (DW + pBR322 plasmid DNA)
Lane 2: Fenton's reagent (FR) + pBR322 plasmid DNA
Lane 3: pBR322 plasmid DNA + FR + test compound (10 µg/ml),
Lane 4: pBR322 plasmid DNA + FR + test compound (50 µg/ml),
Lane 5: pBR322 plasmid DNA + FR + test compound (70 µg/ml) and
Lane 6: pBR322 plasmid DNA + FR + test compound (100 µg/ml)

Table 1
Antigenotoxic effect of compound **1** from leaves of *Anthocephalus cadamba* against 4NQO and AFB1 in SOS chromotest using *E. coli* PQ37 tester strain.

Treatment	Dose (μg/ml)	β-Galactosidase units Mean ± SE	Alkaline phosphatase units Mean ± SE	*R*	Induction factor (IF)
4NQO (direct-acting mutagen)					
Control (non treated cells)		6.23 ± 0.55	29.17 ± 1.99	0.21	1.00
Positive control (4NQO)	20 μg/ml	46.74 ± 4.19	16.57 ± 0.66	2.82	13.43
Negative control (compound **1** only)	10	4.52 ± 0.24	23.72 ± 2.30	0.19	0.90[a]
	30	4.38 ± 0.29	24.34 ± 2.08	0.18	0.86[a]
	100	4.44 ± 0.28	26.43 ± 2.01	0.17	0.81[a]
	300	4.92 ± 0.19	24.06 ± 1.22	0.20	0.95[a]
	1000	5.10 ± 0.18	23.76 ± 1.67	0.21	1.00[a]
4NQO + compound **1**	10	30.58 ± 3.83	12.65 ± 0.45	2.42	11.52
	30	34.34 ± 2.95	12.60 ± 0.49	2.73	13.00
	100	33.86 ± 2.92	14.32 ± 1.62	2.36	11.24[a]
	300	33.89 ± 2.84	16.09 ± 1.44	2.11	10.04[a]
	1000	31.95 ± 6.21	16.03 ± 0.93	1.99	9.48[a]
Aflatoxin B1 (S9-dependent mutagen)					
Control (non treated cells)		10.42 ± 0.75	61.92 ± 5.04	0.17	1.00
Positive control (aflatoxin B1)	20 μg/ml	97.42 ± 2.99	60.87 ± 5.19	1.60	9.41
Negative control (compound **1** only)	10	10.53 ± 0.85	62.57 ± 3.16	0.17	1.00[a]
	30	11.42 ± 0.19	61.13 ± 3.98	0.19	1.12[a]
	100	8.93 ± 1.29	60.73 ± 2.62	0.15	0.88[a]
	300	9.03 ± 1.03	61.00 ± 5.48	0.15	0.88[a]
	1000	9.48 ± 0.51	67.18 ± 5.52	0.14	0.82[a]
Aflatoxin B1 + compound **1**	10	61.10 ± 9.04	59.18 ± 6.71	1.04	6.12[a]
	30	61.27 ± 6.48	57.55 ± 5.18	1.06	6.24[a]
	100	57.13 ± 8.15	56.98 ± 5.02	1.00	5.88[a]
	300	51.65 ± 4.71	69.77 ± 10.6	0.74	4.35[a]
	1000	45.68 ± 4.06	68.45 ± 9.59	0.67	3.94[a]

R, β-galactosidase units/alkaline phosphatase units; IF, Rc/Ro.
[a] Level of statistical significance: $p \leq 0.05$ with respect to 4NQO and Aflatoxin B1.

through isolation, characterization and preclinical evaluation for their development as chemopreventive agents (Lippman and Hong, 2002; Gupta, 2007). Evaluation of antimutagenic activities of natural products becomes necessary, as there is concordance between antimutagenicity and anticarcinogenicity (Maron and Ames, 1983; El-Sayed et al., 2007). Antimutagenic studies of botanical extracts form the foundation for selecting the lead extracts for long term and costly *in vivo* chemoprevention investigations together with the separation and chemical structural elucidation of possible active compounds (EI-Sayed et al., 2013).

In the SOS chromotest, it was ascertained that different concentrations of compounds **1** and **2** added to the indicator bacteria were not genotoxic as the induction factor induced by the tested doses was below 1.5. Table 1 and Fig. 5 showed that at a concentration of 10 μg/ml, compound **1** inhibited the genotoxicity of AFB1 (IF = 9.41) by 39.12% which dose dependently increased and at a concentration of 1000 μg/ml, it showed an inhibition of 65.04%. Similarly, compound **1** inhibited the induction factor of 4NQO (IF = 13.43) by 15.37% at a concentration of 10 μg/ml and 31.78% at 1000 μg/ml. As seen from Table 2 and Fig. 5, compound **2** did not effectively modulated the genotoxicity of 4NQO and AFB1. Compound **2** reduced the induction factor of 4NQO (IF = 10.62) and AFB1 (IF = 8.80) by 37.11% and 47.05% at highest tested concentration of 1000 μg/ml respectively.

Epicatechins are one of the components of tea polyphenols and in a report by Ito et al. (1989), protective effects of hot water green tea extracts were evaluated against the AFB1-induced chromosomal aberrations in bone marrow cells. Mutagenic effects might be inhibited by catechins due to flavanol–mutagen adduct formation (Stich, 1991). Kuroda (1996) reported the bioantimutagenic activity of catechins against the direct-acting mutagen

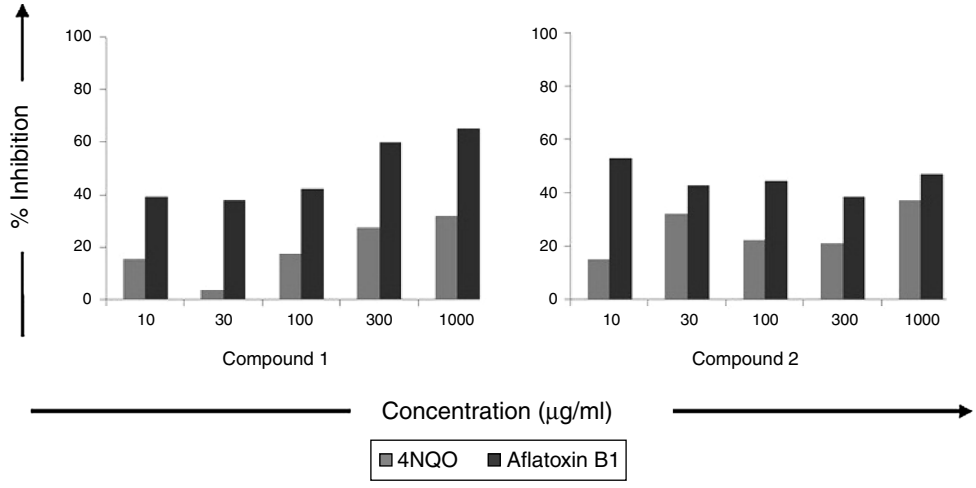

Fig. 5. Effect of compounds **1** and **2** from *Anthocephalus cadamba* leaves on genotoxicity induced by 4NQO and AFB1 in SOS chromotest using *E. coli* PQ37 tester strain.

Table 2
Antigenotoxic effect of compound **2** from leaves of *Anthocephalus cadamba* against 4NQO and AFB1 in SOS chromotest using *E. coli* PQ37 tester strain.

Treatment	(μg/ml)	β-Galactosidase units Mean ± SE	Alkaline phosphatase units Mean ± SE	*R*	Induction factor (IF)
4NQO (direct-acting mutagen)					
Control (non treated cells)		5.71 ± 1.14	26.72 ± 1.19	0.21	1.00
Positive control (4NQO)	20 μg/ml	56.16 ± 6.19	25.17 ± 1.55	2.23	10.62
Negative control (compound **2** only)	10	4.16 ± 0.74	19.20 ± 3.74	0.22	1.04[a]
	30	3.92 ± 0.72	19.62 ± 3.54	0.20	0.95[a]
	100	5.01 ± 0.53	19.31 ± 3.25	0.26	1.24[a]
	300	5.38 ± 0.39	20.05 ± 3.59	0.27	1.29[a]
	1000	6.20 ± 0.62	19.96 ± 3.67	0.31	1.48[a]
4NQO + compound **2**	10	43.24 ± 5.13	22.59 ± 2.35	1.91	9.10
	30	41.01 ± 6.15	22.67 ± 2.17	1.81	8.62
	100	35.26 ± 4.24	22.32 ± 2.21	1.58	7.52
	300	40.67 ± 5.80	23.15 ± 2.34	1.78	8.48
	1000	37.73 ± 7.02	25.46 ± 2.67	1.48	7.05
Aflatoxin B1 (S9-dependent mutagen)					
Control (non treated cells)		11.92 ± 0.21	78.13 ± 3.18	0.15	1.00
Positive control (aflatoxin B1)	20 μg/ml	95.80 ± 1.34	72.63 ± 2.69	1.32	8.80
Negative control (compound **2** only)	10	11.08 ± 0.99	74.63 ± 2.13	0.15	1.00[a]
	30	11.62 ± 0.35	76.77 ± 1.81	0.15	1.00[a]
	100	12.92 ± 0.43	76.10 ± 4.41	0.17	1.13[a]
	300	11.15 ± 0.18	76.57 ± 1.82	0.15	1.00[a]
	1000	10.58 ± 0.32	79.52 ± 0.67	0.13	0.87[a]
Aflatoxin B + compound **2**	10	56.12 ± 2.51	79.83 ± 3.26	0.70	4.67[a]
	30	62.13 ± 1.37	75.40 ± 1.99	0.82	5.47[a]
	100	60.30 ± 6.34	75.40 ± 1.98	0.80	5.33[a]
	300	66.38 ± 3.91	76.35 ± 2.24	0.87	5.80[a]
	1000	59.45 ± 3.22	77.43 ± 1.83	0.77	5.13[a]

R, β-galactosidase units/alkaline phosphatase units; IF, Rc/Ro.
[a] Level of statistical significance: $p \leq 0.05$ with respect to 4NQO and Aflatoxin B1.

i.e. 4NQO. Matsumoto et al. (1996) and Weisburger et al. (1997) reported that epicatechins modulate the activity of detoxifying enzymes *i.e.* reduction of cytochrome P450 and increase in phase II enzymes. Bhouri et al. (2011) reported the antigenotoxicity of two flavonoids, kaempferol 3-*O*-β-isorhamninoside (K3O-ir) and rhamnocitrin and 3-*O*-β-isorhamninoside (R3O-ir) against the genotoxicity induced by nitrofurantoine and aflatoxin B1. K3O-ir and R3O-ir reduced the genotoxicity of aflatoxin B1 significantly by 96.64% and 90.26%, respectively at the highest tested concentration of 10 μg/ml. Flavonoids can also act as desmutagens by directly interacting with mutagens and inactivating them (Heo et al., 1994). Antigenotoxic potential of apigenin (flavone) against mitomycin C induced genotoxic damage in mouse bone marrow cells was studied by Siddique and Afzal (2009). Results of the study demonstrated that apigenin effectively diminished the genotoxicty of mitomycin C as reflected from decrease of sister chromatid exchanges (SCEs) and chromosomal aberrations in mouse bone marrow cells. Hayder et al. (2004) reported the antigenotoxic activity of extracts from *Myrtus communis* and all the extracts were found effective against the genotoxicity of AFB1 and nifuroxazide and they attributed the potential of the tested extracts toward antigenotoxicity to the presence of flavonoids, coumarins and tannins.

Antiproliferative and *in vitro* COX-2 inhibitory activity

Only compound **2** exhibited cytotoxicity against COLO 205 cell line with percent inhibition of 52.06% at 500 μg/ml (Fig. 6). However both the compounds were found to be ineffective against HeLa cell line. Procyanidins (flavonoids)-rich grape seed extract showed growth inhibitory effect and induction of apoptotic cell death in a human prostate carcinoma DU145 cell line (Agarwal et al., 2002). Wang et al. (1999) investigated the mechanism of induction of apoptosis by apigenin, myricetin, quercetin and kaempferol in HL-60 leukemia cells. Apigenin was found to be most potent in reducing the cell viability with IC_{50} of 50 μM. Park and Min (2011) reported that quercetin inhibited invasion and proliferation of glioma cells by downregulation of phospholipase D1, a regulator of cell proliferation and tumorigenesis. Compound **1** from *A. cadamba* leaves inhibited the COX-2 by 17.79% at concentration of 1 μM whereas compound **2** showed 25.64% inhibition at same concentration. Compound **2** was found effective against COLO-205 and this antiproliferative activity of the compound may partly be due to the inhibition of COX-2. Overexpression of COX-2 has been observed in colon tumors (Kawamori et al., 1998; Crofford, 1997; Roelofs et al., 2014). Therefore specific COX-2 inhibitors could potentially serve as chemopreventive agents. Ye et al. (2004) investigated the anticancer activity of genistein on human oral squamous carcinoma line (SCC-25). Genistein inhibited the growth of oral squamous carcinoma cells with IC_{50} of approximately 200 μM *via* G2/M arrest. Genistein at a concentration of 0.1 μM effectively decreased the expression of COX-2.

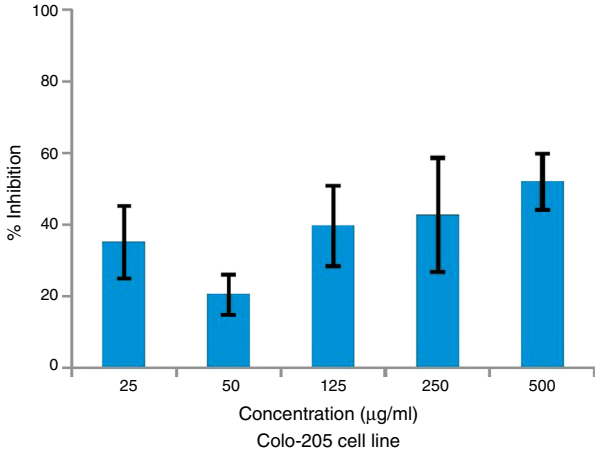

Fig. 6. Growth inhibitory activity of compound **2** on Colo-205 by MTT assay.

Conclusions

The isolated phytochemicals from *A. cadamba* have the potential to alleviate the genotoxicity of environmental mutagens/carcinogens. These have also been found to possess significant antioxidant activity greater than that of standard compound rutin. The results clearly show the chemopreventive potential of *A. cadamba* phytochemicals and can be a promising natural source in health and medicine.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Authors' contributions

MC carried out extraction, isolation and major bioactivity part of present work. MK and MC collectively conducted cytotoxic and COX-2 inhibitory activities along with statistically analysis of the data. US, NK and BS carried out structural elucidation and characterization of isolated compounds. SK designed, supervised and critically checked the manuscript. All authors read and approved the final version of manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2016.02.007.

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