

# Inhibition of H<sub>2</sub>O<sub>2</sub>-induced DNA damage in single cell gel electrophoresis assay (comet assay) by castasterone isolated from leaves of centella asiatica

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Received 10 December 2009; revised 16 February 2010; accepted 20 February 2010.

## ABSTRACT

Brassinosteroids (BRs) are a large group of polyhydroxy steroids, which regulate numerous aspects of plant growth and development, including stem elongation, leaf bending, tracheary element differentiation, stress protection and photomorphogenesis. Recent studies indicate antigenotoxic and anticancerous activities of these compounds. The role of natural BRs in H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide)-induced DNA damage in human lymphocytes is still unknown. The present study reports the presence of Castasterone from leaves of *Centella asiatica*, an important medicinal herb commonly used as a memory enhancer and immunomodulator. CA50 fraction isolated from *Centella asiatica* was characterized as Castasterone by electrospray ionization mass spectral data with standard Castasterone. An attempt has been made to study antigenotoxic activity of the isolated Castasterone against H<sub>2</sub>O<sub>2</sub>-induced DNA damage in human blood lymphocytes using Single cell gel electrophoresis assay (Comet Assay). Castasterone at 10<sup>-9</sup> M concentration proved to be effective in diminishing the DNA damage by 89.42 %.

**Keywords:** Brassinosteroids; Castasterone; Comet Assay; Hydrogen Peroxide

## 1. INTRODUCTION

In living system, oxidative stress results in the production of reactive oxygen species (ROS) like superoxide radical (O<sub>2</sub><sup>-</sup>), hydroxyl radical (HO<sup>-</sup>) and hydrogen per-

oxide (H<sub>2</sub>O<sub>2</sub>). H<sub>2</sub>O<sub>2</sub> in Fenton reaction is spontaneously converted to the highly reactive hydroxyl radicals (HO<sup>-</sup>). These hydroxyl radicals oxidize proteins, lipids and nucleic acids leading to even mutations at the cellular level [1]. Several plant hormones are implicated in modulating the response to oxidative stress like ethylene [2], abscisic acid [3], auxins and plant steroids [4]. Brassinosteroids are a group of naturally occurring plant hormones, which are structurally similar to animal steroid hormones. They influence diverse physiological processes by regulating the expression of genes like their animal counterparts [5]. Recent studies indicate antiviral activities of BRs against various viruses, like herpes simplex virus type I (HSV1), arena virus, measles virus and vesicular stomatitis virus [6-8]. The treatment of BRs to these viruses was 10-18 folds more effective than ribavirin towards HSV-I and arenavirus. It has further been reported that 24-epibrassinolide can increase the mitochondrial membrane potential, reduce intercellular antibody levels, increase the proportion of cells in G<sub>0</sub>/G<sub>1</sub> phase, reduce the population of cells in s-phase and increase the population of viable hybridoma mouse cells at subnanomolar concentrations [9]. Anticancerous activities of 28-homocastasterone and 24-epibrassinolide were studied in several normal and cancer cell lines. The anticancer and antiproliferative activities have been documented very recently [10]. The BRs used showed high cytotoxic activity in breast (MCF-7/MDA-MB-468) and prostate cancer cell lines (LNCaP/DU-145) [11].

*Centella asiatica* (L.) (Family Apiaceae) commonly known as urban herb regarded as rasayana or rejuvenating herb reputed to increase intelligence and memory in Ayurvedic medicines. The methanol extracts of whole plants of *Centella asiatica* showed a significant increase in the phagocytic index and total WBC count thereby showing immunomodulatory activity. [12] isolated a

water soluble arabinogalactan, HBN and traced remarkable immunoenhancing activities on T-and B-lymphocytes *in vitro* and *vivo* tests. The antioxidative properties of *Centella asiatica* were evaluated by [13,14]. The *C. asiatica* extract has a chemopreventive effect [15]. [16] studied the healing effects of *C. asiatica* when orally administered to rats with acetic acid induced gastric ulcers, It reduced the size of ulcers in dose-dependent manner. Chemical studies reveal that triterpene saponins Asiaticoside and Madecassoside are the main active constituents of *Centella asiatica*. The other saponins and triterpene acids present in this plant are brahmoside, brahmnoside, brahmnic acid, isobrahmic acid, betulic acid, centelloside and cetillic etc. The presence and role of brassinosteroids in this plant is yet to be studied. The present study was therefore planned to study the presence of BRs and inhibition of H<sub>2</sub>O<sub>2</sub>-induced DNA damage by Castasterone isolated from *Centella asiatica* which is the first report in this direction.

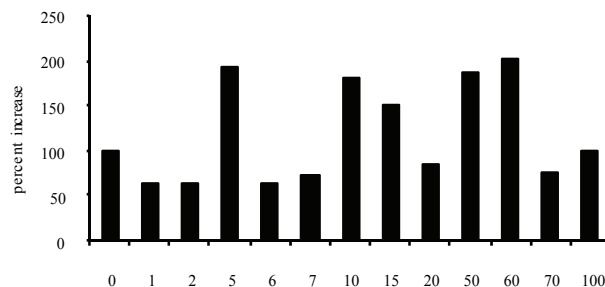
## 2. MATERIALS AND METHODS

### 2.1. Extraction and Purification of Brassinosteroids

Study material for the present investigation included leaves of *Centella asiatica* procured from Dehradun (M/s Gautam globals, Dehradun, India). Fresh leaves of *Centella asiatica* (2 kg) were homogenized and percolated with 80% methanol (3 × 1000 ml). The combined methanol extract was dried under vacuum using rotary evaporator (Strike 202, Stereoglass, Italy). 80% methanol extract (449.6 g) was partitioned between chloroform and water. Chloroform extract was then partitioned between 80% methanol and hexane. The resulting 80% methanol extract (28.3 g) was partitioned between ethyl acetate and distilled water. The ethyl acetate fraction (20 g) was dried and subjected to silica gel (60-120 mesh) column chromatography with step-gradient elution from 0, 1, 2, 3, 4, 5, 6, 7, 10, 15, 20, 50, 100% (each 500-1000 ml). All the fractions were subjected to radish hypocotyl bioassay with the aim to find the bioactive fraction. Four fractions CA5, CA10, CA50 and CA60 were found to be active (**Figure 1**). CA50 fraction was directly subjected to ESI-MS and MS/MS analysis (**Figures 2(a), 3(a)**).

### 2.2. Radish Hypocotyl Bioassay

The bioactivity of isolated fractions was determined using intact plants of *Raphanus sativus* as described by [17]. 5 days-old seedlings were placed into the test solutions (0.03 ml of fraction diluted with distilled water to get the final volume 3 ml). 3 ml solution was poured in each petriplate containing radish seedlings and kept in



**Figure 1.** Biological activity of fractions of *Centella asiatica* in Radish hypocotyls bioassay after silica gel column chromatography.

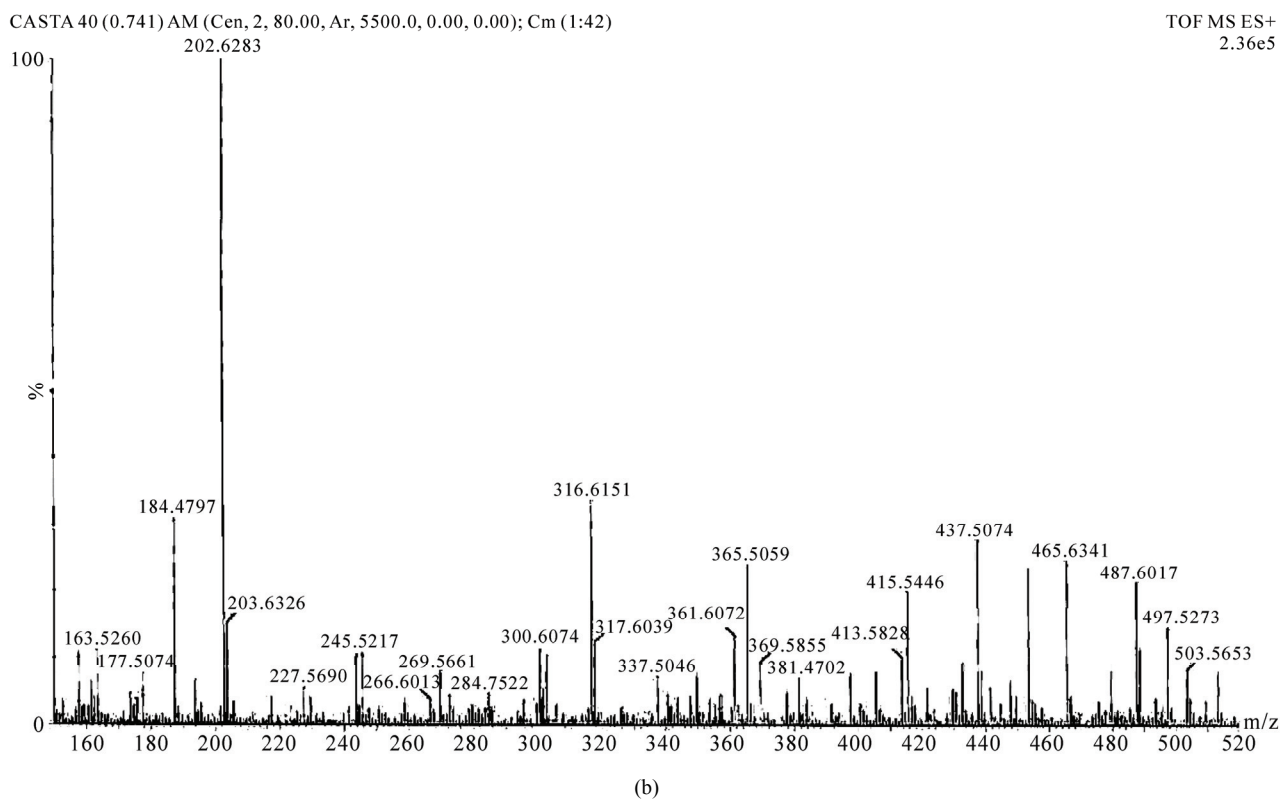
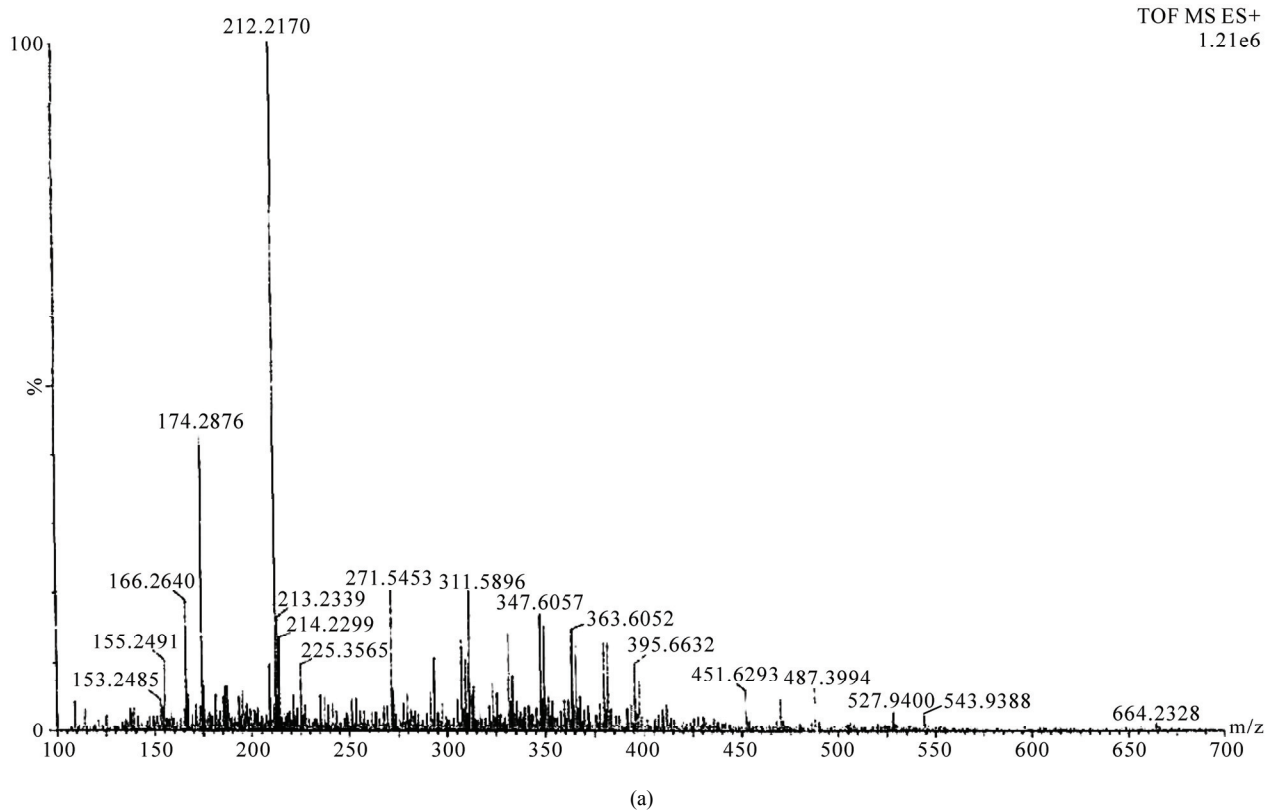
the dark for 24 h at 25 ± 2°C. After 24 h, the length of hypocotyls were measured and compared to control. Percent increase over control was calculated.

### 2.3. Electrospray Ionization Mass Spectrometry of CA 50 Fraction

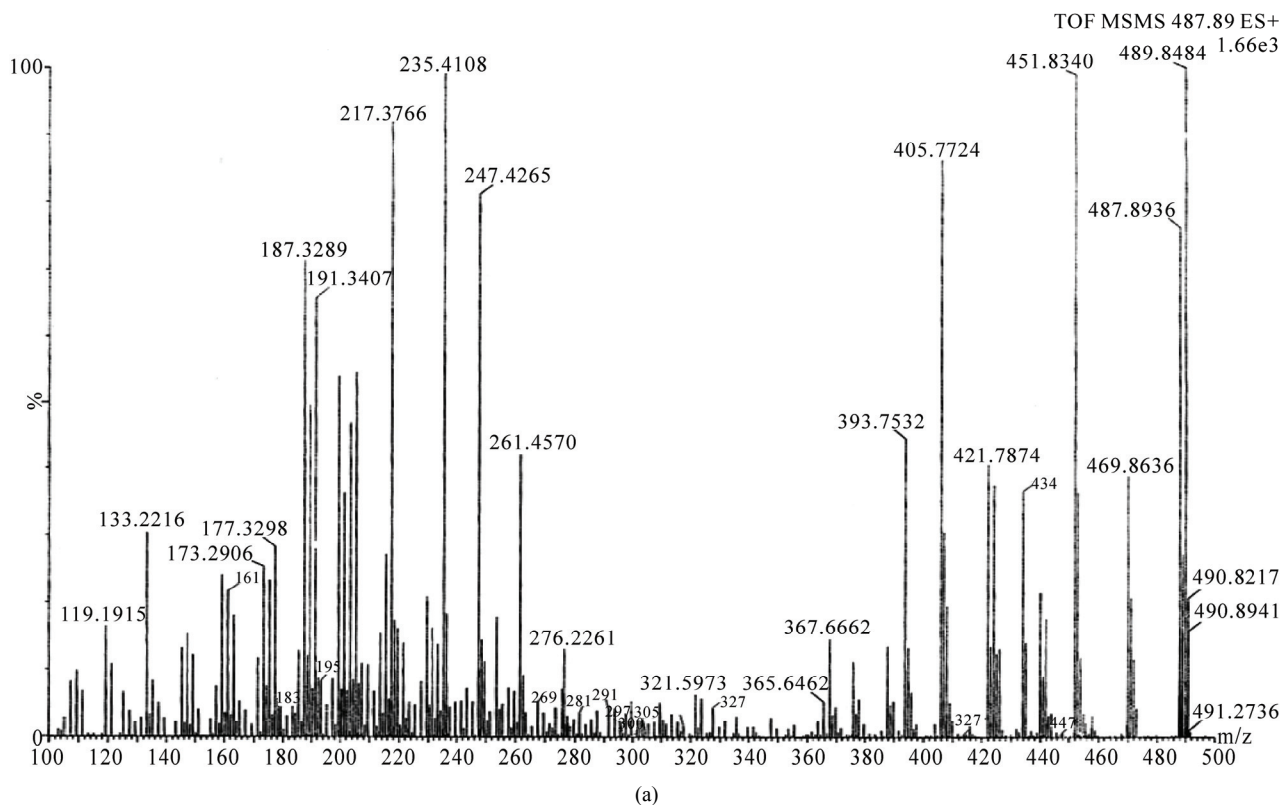
ESI-MS analysis of CA50 and standard Castasterone was carried out by the addition of 10 µl of concentrated aqueous formic acid solution to the sample mixture to a total volume of 1000 µl making 0.1% as final concentration. ESI-QTOF-MS was performed in positive ionization mode in QTOF Mass Spectrometer (Micromass, Manchester, UK). The general conditions were: Source temperature of 280°C, capillary voltage of 2.1 kV and cone voltage of 23 V. ESI-MS was performed by direct infusion with a flow rate of 10 µl/min using a syringe pump and mass spectra were acquired and accumulated over 60 s. MassLynx 4.0 (Waters, Manchester, UK) was used for data analysis. Tandem mass spectrometry of single molecular ion in the mass spectra was performed by mass-selecting the ion of interest, which was in turn submitted to 15-35 eV collisions with argon in the collision quadrupole.

### 2.4. Comet Assay

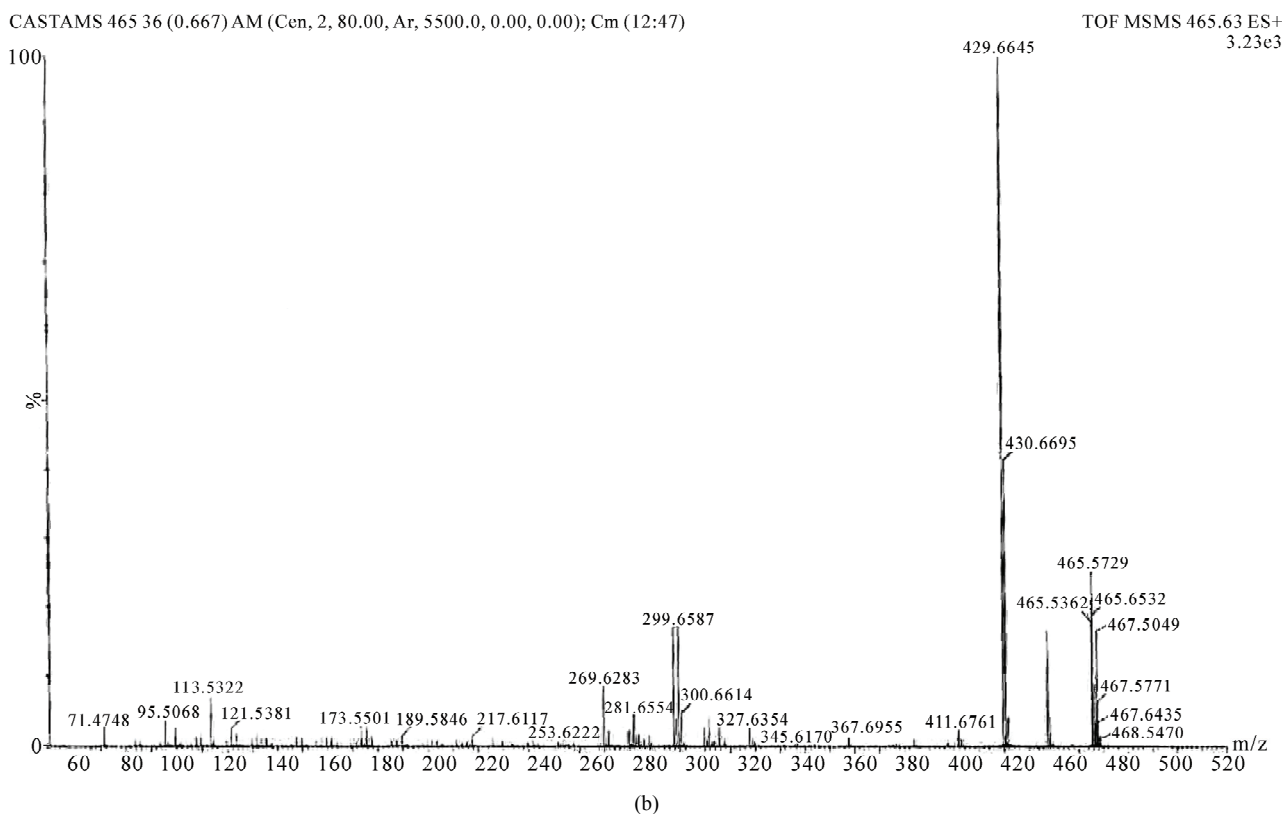
DNA damage was determined by alkaline single cell microgel electrophoresis (comet assay) assay following the method proposed by [18] with minor modifications as suggested by [19]. Heparinized blood samples were obtained by venipuncture from a non-smoking, healthy male donor aged 30-40 years. Lymphocytes were isolated by the method of [20] and mixed with equal volume of Phosphate Buffer Saline (PBS) pH 7.2. This mixture was then overlaid to double volume of Histo-paque 1077 and centrifuged at 1500 rpm for 20 minutes. The layer containing lymphocytes was aspirated very carefully with the help of pasture pipette. The lymphocytes were diluted in PBS and centrifuged at 2000 rpm for 15 min. The supernatant was discarded and pellet was again suspended in PBS and centrifuged at 2000 rpm



**Figure 2.** (a) ESI-QTOF-MS analysis of Castasterone fraction (CA50) isolated from *Centella asiatica*; (b) ESI-QTOF-MS of Standard Castasterone.



CASTAMS 465.36 (0.667) AM (Cen, 2, 80.00, Ar, 5500.0, 0.00, 0.00); Cm (12:47)



**Figure 3.** (a) ESI-QTOF-MSMS analysis of Castasterone fraction (CA50) isolated from *Centella asiatica*; (b) ESI-QTOF-MSMS analysis of Standard Castasterone.

Human blood lymphocytes suspended in 1 ml PBS, were incubated in a shaking water-bath for 30 minutes at 37°C with  $5 \times 10^{-5}$  M  $H_2O_2$  in the presence of different concentrations of CA50 fraction tested in duplicate. To evaluate the extent of DNA damage, 100 randomly selected cells were analysed from each sample by Nikon Epifluorescent Microscope (Nikon Eclipse E200) connected to a digital camera. Imaging was performed by using a computerized image analysis system (Lucia Comet Assay Software 4.8 of Laboratory Imaging Ltd.) which acquires images, computes the integrated intensity profile for each cell, estimates the comet cell components (head and tail) and evaluates a range of derived parameters. These include % Head DNA, % Tail DNA and tail moment (an index of DNA damage that considers both the tail length and fraction of DNA in comet tail).

The Antigenotoxic activity of CA50 was expressed by percent decrease of Tail moment:

$$\text{Inhibition (\%)} = a - b/a - c \times 100$$

a = Tail moment induced by  $H_2O_2$  (positive control).

b = Tail moment of the fraction in the presence of  $H_2O_2$ .

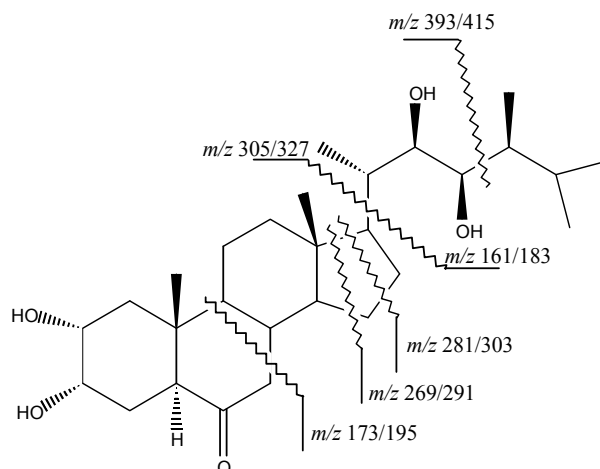
c = Tail moment of the negative control.

### 3. STATISTICAL ANALYSIS

The results were obtained as the mean and standard error of three experiments. The data in all the experiments were analyzed for statistical significance using analysis of variance (one-way ANOVA). The difference among average values was compared by high-range statistical domain (HSD) using Tukey's Test [21].

#### 3.1. Results and Discussion

CA50 fractions was isolated and characterized as castasterone by ESI-QTOF-MS/MS analysis. Electrospray ionization mass spectroscopy of standard castasterone and CA50 fraction (**Figures 2(a)** and **2(b)**) showed the pseudomolecular mass ion peaks at  $m/z$  465  $[M + H]^+$  and 487  $[M + Na]^+$  corresponded to the molecular weight 464  $[M]^+$  and molecular formula as  $C_{28}H_{48}O_5$ . ESI-QTOF-MS/MS analysis of  $m/z$  465/487 revealed similar kind of fragmentations for CA50/ standard castasterone (**Figures 3(a)** and **3(b)**). The fragments at  $m/z$  447/469, 429/451 were observed due to the sequential loss of two  $H_2O$  molecules. The fragments at  $m/z$  393/415 and 305/327 were observed due to the  $C_{23}$ - $C_{24}$  and  $C_{17}$ - $C_{20}$  bond fissions. Other fragments were detected at  $m/z$  411/433, 297/319, 281/303, 269/291, 173/195 and 161/183. **Figure 4** shows the important fragmentation pattern of castasterone. We report for the first time, the presence of Castasterone in *Centella asiatica*. The



**Figure 4.** Structure of Castasterone and major mass fragmentations.

presence of brassinosteroids in medicinal plant like *Centella asiatica* suggests a possible medicinal application of these compounds. Brassinosteroids are found in gymnosperms, monocotyledonous and dicotyledonous plants, and in algae. Studies [22-24] confirmed that BRs are obligatory plant constituents, the highest concentration being found in the reproductive organisms and in growing tissues (pollen, immature seeds and shoots).

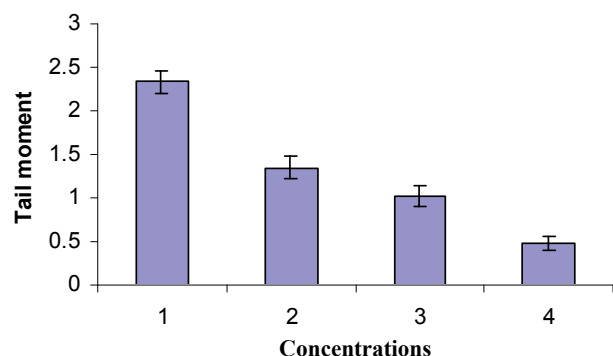
$H_2O_2$ -induced DNA damage was studied using various parameters. These parameters were measured using Lucia Comet Assay Software. Percent inhibition was calculated on the basis of comet evaluation *i.e.* Tail moment. The observations made on various parameters of DNA indicated that  $10^{-9}$ ,  $10^{-10}$  and  $10^{-11}$  M concentrations of Castasterone were not toxic as no significant change was noticed when compared with negative control *i.e.* distilled water.

Percent head DNA in the treatments of Castasterone revealed no significant change when compared with negative control *i.e.* only distilled water. The  $H_2O_2$  treatment however reduced %Head DNA. But supplementation of Castasterone with  $H_2O_2$  to lymphocytes revealed an observable enhancement in % Head DNA. It increases from positive control (83.08%) to (93.66%) at  $10^{-9}$  M concentration (**Table 1**). Similar observations were made for % Tail DNA and Tail moment. BRs treatments showed amelioration of toxicity. % Tail DNA decreased significantly with the increase in concentration of castasterone. A significant decrease in Tail moment was observed with the increase in the concentration of castasterone (**Figure 5**). It varied from 1.35 ( $10^{-11}$  M), 1.02 ( $10^{-10}$  M), 0.48 ( $10^{-9}$  M). Percent inhibition was maximum in  $10^{-9}$  M concentration *i.e.* 89.42% (**Table 1**). The reports obtained on the toxicity of BRs suggested that they do not have negative influences in mammals,

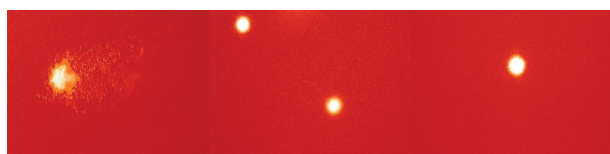
**Table 1.** Inhibition of H<sub>2</sub>O<sub>2</sub>-induced DNA damage in Human blood lymphocytes by CA50 fraction isolated from *Centella asiatica* using Comet assay.

Treatment	Dose concentration	%Head DNA (Mean ± SE)	% Tail DNA (Mean ± SE)	Tail moment (Mean ± SE)	% Inhibition
Negative control	D.W	96 ± 0.32	4.0 ± 0.32	0.30 ± 0.02	
	10 <sup>-11</sup> M	92.28 ± 2.06	7.72 ± 0.40	0.24 ± 0.03	
Castasterone	10 <sup>-10</sup> M	96.09 ± 0.41	3.91 ± 0.41	0.26 ± 0.03	
	10 <sup>-9</sup> M	95.82 ± 0.56	4.22 ± 0.58	0.26 ± 0.03	
Positive control	H <sub>2</sub> O <sub>2</sub> (50 μM)	83.02 ± 0.72	16.98 ± 0.72	2.34 ± 0.13	
	10 <sup>-11</sup> M	88.68 ± 1.06*	11.32 ± 1.06*	1.35 ± 0.13*	47.14%
Castasterone + H <sub>2</sub> O <sub>2</sub> (50μM)	10 <sup>-10</sup> M	91.69 ± 0.8*	8.28 ± 0.81*	1.02 ± 0.11*	63.46%
	10 <sup>-9</sup> M	93.66 ± 0.98*	6.34 ± 0.98*	0.48 ± 0.07*	89.42%

\*indicates significant values at p ≤ 0.05.



**Figure 5.** Effect of Castasterone isolated from *Centella asiatica* on the genotoxicity induced by Hydrogen peroxide ( $5 \times 10^{-5}$  M) in human lymphocytes using comet assay. 1 = treatment with H<sub>2</sub>O<sub>2</sub>. 2-4 = treatment with different concentrations of Castasterone.



(a) (b) (c)

Digital images illustrating the inhibition of DNA damage by Castasterone in the Comet assay (a) +ve control *i.e.* H<sub>2</sub>O<sub>2</sub> (b) -ve control *i.e.* Castasterone only (c) Castasterone + H<sub>2</sub>O<sub>2</sub>.

water organisms, soil microbiological processes and plants [25]. Mutagenic studies carried out at the Scientific Research Center of Toxicological and Hygienic Regulation of Biopreparations of Russia showed that Ames test, with or without metabolic activation, was negative with the tester strains of *Salmonella typhimurium* TA1534, TA1537, TA1950, TA98 and TA100

[25]. Antigenotoxic properties of EBL isolated from *A. marmelos* had also been studied by [26].

Reactive oxygen species can damage the normal cellular functions and can cause atherosclerosis in vessels or malignant growth in other tissues and ageing processes [27]. The lymphocytes when treated with H<sub>2</sub>O<sub>2</sub> showed the significant DNA damage. However this damage was ameliorated significantly by the simultaneous application of different concentrations of this BR. The H<sub>2</sub>O<sub>2</sub> stress protective properties of BRs in human lymphocytes are the first such study carried out with plant steroids. In the present study, the protective effect observed against the ROS may in part be responsible to the anticancer activity of brassinosteroids reported by some workers [11,28]. In the studies carried out by [29] three types of 5 $\alpha$ -androstane and ergostane analogues of brassinolide, containing a fluorine atom in either the 3 $\alpha$  or the 5 $\alpha$  positions or in 3 $\alpha$  or the 5 $\alpha$  positions, were prepared using standard operations. The 5 $\alpha$  fluorine was found to effect chemical reactivity as well as physical properties of the products. Cytotoxicity of the products was studied using human normal and cancer cell lines with 28-homocastasterone as positive control and their brassinolide type activity was established using the bean second-internode test with 24-epibrassinolide as standard. The equivalence of F and OH groups was observed in some of the active compounds. Ergostane derivatives were most active in the anticancer activity while androstane derivatives were active in brassinolide type activity. Brassinolide was found to induce a time and concentration dependent cytotoxicity in androgen-independent human prostate cancer in PC-3 cells. The mode of cell death appeared to be predominately apoptosis. Western blot studies indicated that treatment with brassinolide triggered a time dependent decrease in the expression of

antiapoptotic protein Bcl-2. 24-epibrassinolide and 28-homoCS were found to inhibit the growth, at micromolar concentrations, of several human cancer cell lines without affecting the growth of normal cells [10]. Studies carried out by Malikova *et al.* (2008) indicate that BRs may prove to be promising leads for the development of new generation of anticancer drugs. Various animal steroids have been found to exhibit antioxidant properties [30]. BRs have also been reported to regulate antioxidative defence system of plants under stress conditions [31]. The reduction in DNA damage indicates amelioration of oxidative stress generation by H<sub>2</sub>O<sub>2</sub> in lymphocytes. The tissues are protected from oxidative damage by variety of mechanism including antioxidants and antioxidative enzymes, repair enzymes and growth regulators. Further studies are needed to understand the mechanism of protective effect of these steroids in animal system which opens a field of study on possible medical applications of these plant steroids.

#### 4. ACKNOWLEDGEMENTS

This work is supported by grants from University Grants Commission, Government of India. Dr. P.S. Ahuja, Director, Institute of Himalayan Bioresource Technology (IHBT) Palampur (HP) is duly acknowledged for providing necessary lab facilities. Dr. Chalev Pachthong, Department of Chemistry, Faculty of Science and Technology Kanchanaburi Rajabhat University, Kanchanaburi, Thailand is also acknowledged for his kind gift of Castasterone.

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