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Laboratory note

Anti-genotoxic hydrazide from *Crinum defixum*

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ABSTRACT

Crinum defixum Ker-Gawl popularly known as Bon-naharu (meaning wild garlic) in Assam. It is found abundantly growing wild on riverbanks of Dhansiri River in Golaghat District of Assam. It is used as ethnomedicine in this part of India for a number of ailments.
Bioassay guided chemical investigation of the bulbs of *Crinum defixum* Ker-Gawl afforded to isolate a new hydrazide derivative and its structure was determined as (*E*)-*N*′-[(*E*)-2-butenoyl]-2-butenoylhydrazide by spectroscopic methods. The compound was assayed for anti-genotoxic activity by onion root tip assay (by observing different types of chromosomal aberrations such as chromosomal bridges, stickiness, delayed anaphase, polyploidy and vagrant chromosome).
The phyto-compound was found to have anti-genotoxic activity and imparted a clear dose dependent protective effect against the genotoxic effect of H_2O_2. Further, the compound seems to be more effective against clastogenic aberrations than physiological aberration at the highest concentration used (250 ppm).

1. Introduction

The use of herb for their therapeutic or medicinal value is the oldest form of healthcare. Its scientific perspective deals with the search for various active components and understanding its mechanism of action to encourage the medicinal use. The anti-genotoxic assay that reveals the protective effect of plant materials against changes in the genetic material induced by chemical or physical influence is one of the regularly used assays. One of the methods of studying mutagenicity is by chromosomal analysis during mitosis. The root chromosomal aberration assay is an established plant bioassay validated by the International Programme on Chemical safety (IPCS, WHO) and United Nations Environment Programme (UNEP) as an efficient and standard test for chemical screening and in situ monitoring for genotoxicity of environmental substances [1]. In continuation of our search for bioactive secondary metabolite from the flora of the South Eastern sub-Himalayan region of biodiversity hotspot of the Indo-Burma belt [2–6], we have investigated *Crinum defixum* Ker-Gawl (family Amaryllidaceae) and isolated an anti-genotoxic hydrazide derivative from its bulb.

C. defixum Ker-Gawl (family Amaryllidaceae), has a wide geographical distribution from India to Borneo and is common on riverbanks and swampy places in Deccan and Bengal [7,8]. The plant is popularly known as Bon-naharu (meaning wild garlic) in Assam and found abundantly growing wild on riverbanks of Dhansiri River in Golaghat District of Assam. The bulb of this plant is fusiform, stoloniferous base, neck cylindrical. Flowers are sessile, fragrant at night and tinged with red [9]. The bulb is nauseant, emetic, emollient and diaphoretic. It is used in the treatment of burns, whitlow and carbuncle [10]. The bulb is toxic to cattle. In otitis, a few drops of the juice of the leaves are instilled into the ear. The leaves are devoid of the toxic principle. The literature survey revealed that *C. defixum* has shown the presence of caranine, crinamine, crinine, galanthamine, galanthine, haemanthamine and hippestrine [11]. A new alkaloid 5α-hydroxyhomolycorine has also been isolated from *C. defixum* [12]. In this presentation, we wish to describe isolation and characterization of a new hydrazide compound from the bulb of *C. defixum* with anti-genotoxic properties by the onion root tip assay (by observing different types of chromosomal aberrations such as chromosomal bridges, stickiness, delayed anaphase, polyploidy and vagrant chromosome).

2. Result and discussion

2.1. Isolation and structure determination

The dichloromethane extract of the concentrated ethanol extract of the dried and powdered root bulb of *C. defixum*, yielded

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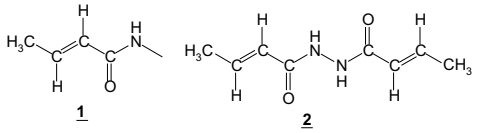
one major compound by bioassay guided investigation. The compound was analyzed as $C_8H_{12}N_2O_2$ by elemental analysis and Electrospray Ionization MS (Bruker Esquire 3000 Iontrap Mass spectrometer) with $[M+H]^+$ and $[M+Na]^+$ at 169 and 191, respectively. In the FTIR spectrum, the strong absorption band at 1705 cm^{-1} and medium strong absorption band at 1656 indicated the presence of α,β-unsaturated amide functional group in the molecule. In the ^1H NMR spectrum recorded at 300 MHz in $CDCl_3$, a doublet of doublet at δ 1.91 integrated to three proton with $J = 1.62$ and 6.96 Hz was assigned to a methyl group at carbon–carbon double bond. Two doublet of quartet at δ 5.84 and 7.11, each having a coupling constant of $J = 15.5$ Hz was assigned to two trans protons attached to a carbon–carbon double bond. Other coupling constants of these signals i.e. $J = 1.62$ Hz (δ 5.84) and $J = 6.95$ Hz (δ 7.11) indicated that these two protons are coupled to the methyl group (δ 1.91). The broad signal at δ 9.81 integrated to one proton was assigned to the amide proton (–CONH–) [13]. Thus, the molecule was found to be very similar to *trans* crotonic acid and apparently ^1H NMR accounts for only the following fragment **1**.



1 **2**

The ^{13}C NMR spectrum of the compound also confirmed the presence of this fragment with a quartet at δ 18.25, two doublets at δ 123.02 and 148.41 and a carbonyl carbon at 172.75. Further, its ^1H and ^{13}C NMR spectra showed no other signals. The quasi-molecular ion $[M+H]^+$ at *m*/*z* 169 and other cluster ion $[M+Na]^+$ at *m*/*z* 191 present in the positive electrospray ionization mass spectrum confirmed that the structure of the molecule as dimer of the above fragment **1**, Therefore, the structure of the molecule was assigned as (*E*)-*N*′-[(*E*)-2-butenoyl]-2-butenoylhydrazide **2**.

Crotonic acid was reported earlier from *Croton tiglium* L. but it was later shown that this was actually structurally related tiglic acid (*E*-2-methyl-2-butenoic acid) [14]. Recently, it was reported that crotonic acid was isolated from carrot seeds as a natural bioactive agent [15]. However, to the best of our knowledge, it is the first report of isolation of an *N*,*N*′-dicrotonyl hydrazide from nature.

2.2. Anti-genotoxic activity

Hydrazide compound **2** was tested for anti-genotoxic effect using the Allium test, a well-established and frequently used assay for the determination of the anti-genotoxic effect of various substances [16].

The cytological effect of H_2O_2 (0.7%) was first examined. It induced more of physiological aberration (c-mitosis, stickiness, delayed anaphase, laggard chromosome and vagrant chromosome) then the clastogenic aberrations (chromosomal bridge and chromosomal breaks) (Fig. 1). It may be possible that cells undergo a state of oxidative stress due to the H_2O_2 effect. Active oxygen forms emerging under the oxidative stress are known to affect the cytoskeleton structure [17]. In their turn, disorders in the cytoskeleton results in disorganization of intracellular transport and oxygen consumption resulting in the intracellular hypertoxia and the active oxygen forms [17]. Disorder in the stability of cytoskeleton proteins under oxidative stress caused the inhibition of mitotic events and resulted in cell death. The effect of hydrazide compound **2** on H_2O_2 induced chromosomal aberrations is summarized in Table 1. Hydrazide compound **2** imparted a clear dose dependent protective effect against the genotoxic effect of H_2O_2 (Fig. 2). In the study the compound seems to be more effective against clastogenic aberrations than physiological aberration showing maximum percentage inhibition of 100 and 91.6%, respectively at the highest concentration used (250 ppm). The reduction in percentage of aberrations in hydrazide compound **2** treated groups after H_2O_2 treatment showed that the compound may have anti-genotoxic effect. In 2002, Shukla and Taneja [18] had also reported anti-mutagenic effect of garlic extract in Swiss albino mice. According to them presence of sulfhydryl compounds implicated for its anti-mutagenic and anti-carcinogenic effects. The mechanism for protection of garlic involves scavenging potentially toxic and mutagenic electrophiles and free radicals and modification of phase II enzymes and I profile that enhance detoxification pathways. Many workers also reported anti-snake venom [19], anti-inflammatory and analgesic [20] and sedative [21] activities of various *Crinum* spp.

The results of the present study are therefore important since they suggested the anti-genotoxic effect of hydrazide compound **2**. Currently, we are also working on the anti-genotoxicity of similar synthetic *N*,*N*′-diacyl hydrazide derivatives. In a recent report

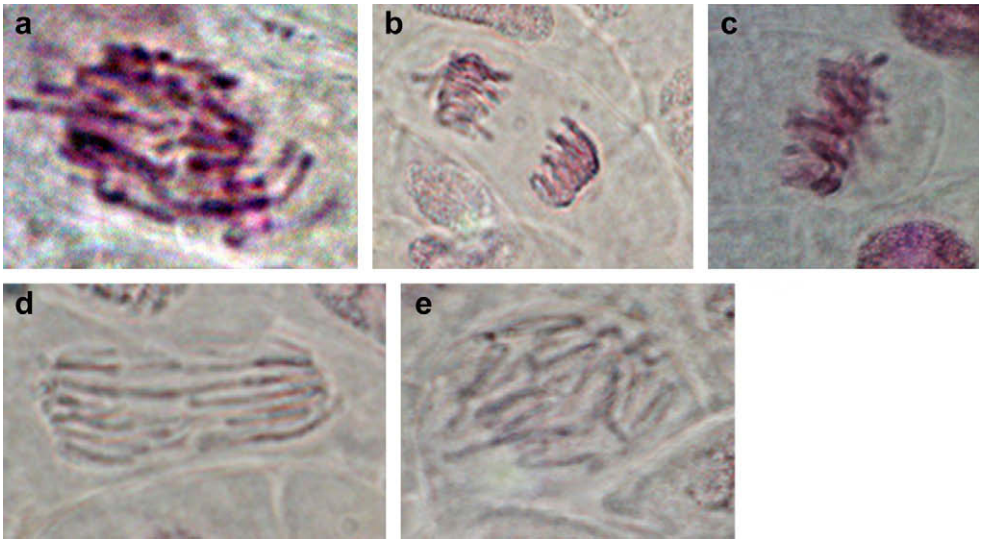


Fig. 1. H_2O_2 induced chromosomal aberration in root tip cells of *A. cepa*. Representative chromosomal breaks (a), vagrant chromosome (b), stickiness (c), chromosomal bridges (d) and delayed anaphase (e) are shown.

Table 1
Effect of (*E*)-*N*′-[(*E*)-2-butenoyl]-2-butenoylhydrazide **2** treatment on H_2O_2 induced genotoxic effect in the root tip of *A. cepa*.

Type of chromatic aberration	*C* −ve	*C* +ve	Concentration of hydrazide compound **2** (ppm)							
			1.95	3.91	7.81	15.62	31.25	62.5	125	250
Physiological aberration										
C-mitosis	3.67 ± 0.9	17.67 ± 0.3	16.33 ± 0.8	13.67 ± 0.8	13.00 ± 0.6[a]	10.67 ± 0.7[a]	8.33 ± 0.9[a]	8.67 ± 0.9[a]	8.00 ± 0.6[a]	4.67 ± 0.9[a]
Stickiness	1.33 ± 0.7	10.33 ± 0.9	11.67 ± 0.3	11.00 ± 0.6	9.67 ± 0.3	9.00 ± 0.6	6.67 ± 0.7[a]	6.33 ± 0.7[a]	5.33 ± 0.9[a]	3.67 ± 0.9[a]
Delayed anaphase	–	5.67 ± 0.9	3.33 ± 0.3[a]	3.33 ± 0.3	2.33 ± 0.3[a]	2.33 ± 0.9[a]	2.00 ± 0.6[a]	0.33 ± 0.3[a]	–	–
Laggard chromosomes	–	7.67 ± 0.7	6.33 ± 0.8	4.67 ± 0.3[a]	0.67 ± 0.3[a]	1.00 ± 0[a]	–	–	–	–
Vagrant chromosomes	–	1.33 ± 0.7	1.33 ± 0.3	1.33 ± 0.3	1.33 ± 0.3	1.33 ± 0.3	–	–	–	–
Total physiological aberration	5	42.67	37.00	34.00	27.00	24.67	17.00	15.33	13.3	8.33
Clastogenic aberration										
Chromatid bridges	–	19.33 ± 0.9	14.33 ± 1.2[a]	14.67 ± 0.7[a]	12.67 ± 0.3[a]	11.00 ± 0.6[a]	6.67 ± 0.7[a]	4.67 ± 0.3[a]	2.67 ± 0.3[a]	1.67 ± 0.7[a]
Chromosomal breaks	5 ± 1.0	22.00 ± 0.6	16.33 ± 1.2	16.00 ± 0.6[a]	15.00 ± 0.6[a]	15.00 ± 0.6[a]	6.33 ± 0.7[a]	5.00 ± 0.6[a]	3.67 ± 0.9[a]	3.33 ± 0.9[a]
Total clastogenic aberration	5	41.33	30.67	30.67	27.67	26.00	13.00	9.67	5.67	5.00
Total aberration	10	84	67.67	64.67	54.67	50.67	30.00	25.00	18.67	13.33

C (−): Negative control; *C* (+): positive control (0.7% H_2O_2); values are mean ± SEM.
[a] $P \leq 0.05$ significantly different from positive control (Student's *t*-test), no significant difference from negative control.

Chabchoub et al. elaborated anti-genotoxicity of pyranotriazolopyrimidine which was synthesized from hydrazine as one of the starting material and it too contains *N*–*N* moiety with neighbouring carbon double bonded with heteroatoms in the molecule [22]. Anti-genotoxicity of these two molecules, hydrazide **2** and pyranotriazolopyrimidine, might be due to this structural fragment. In order to reach to certain conclusion about this subject, however, further research should be performed with different test systems.

3. Experimental

3.1. General experimental procedure

IR spectra were recorded on a Perkin Elmer System 2000 FTIR spectrometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker AVANCE DPX 300 NMR spectrometer in $CDCl_3$ using TMS as the internal standard. (+)-Electrospray Ionization mass spectra (ESIMS) were recorded on Bruker Esquire 3000 system. Silica gel G was used for TLC. All solvents used were distilled prior to use.

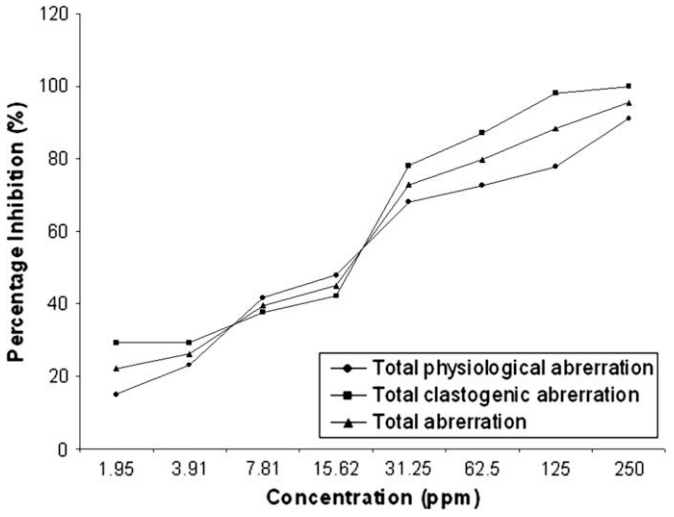


Fig. 2. Percentage inhibition against physiological, clastogenic and total aberrations in (*E*)-*N*′-[(*E*)-2-butenoyl]-2-butenoylhydrazide (Compound **2**) treated groups after H_2O_2 treatment.

3.2. Plant material

C. defixum plants were collected from wild from river bank of Dhansiri River in Golaghat District of Assam in August, 2005. A voucher specimen has been deposited in RRL herbarium (Accession No. RRLJ/227).

3.3. Extraction and isolation of (E)-N′-[(E)-2-butenoyl]-2-butenoylhydrazide **2**

Bulbs of *C. defixum* were collected and dried under shade and powdered. 250 g of dry powdered materials was immersed 95% EtOH for 24 h. Then, it was filtered and the marc was again immersed in 95% EtOH. The process was repeated for three times. The combined extracts were then concentrated under reduced pressure at below 50 °C with a rotary evaporator. The concentrate (20 g) was then dissolved in 200 mL 30% aq. EtOH. The aqueous solution was extracted with hexane (150 mL × 3), CH_2Cl_2 (150 mL × 3) and EtOAc (150 mL × 3). The dried CH_2Cl_2 extract (dry Na_2SO_4) was then distilled under reduced pressure to get a crude extract of 2 g. TLC examination of the crude revealed that it contained a major compound. The compound was then isolated from the crude extract by combined use of column chromatography and prep. TLC (silica gel, solvent system petrol: EtOAc:1:1) as a brown coloured gum (56 mg, yield 0.0224%) characterized as (*E*)-*N*′-[(*E*)-2-butenoyl]-2-butenoylhydrazide **2** by spectroscopic methods.

3.3.1. Spectral data of (E)-N′-[(E)-2-butenoyl]-2-butenoylhydrazide **2**

Gum. IR $\nu_{max}^{thin\ film}$ cm^{-1}: 3409, 2977, 2906, 2855, 1705, 1656, 1446, 1420, 1377, 1287, 1233, 1163, 1046 and 969. ^{1}H NMR spectral data (300 MHz, TMS, $CDCl_3$): δ 1.91 (6H, dd, $J_{1,2} = 6.96$ Hz, $J_{1,3} = 1.62$, H-4 & 4′), 5.84 (2H, dq, $J_{1,3} = 1.62$ Hz, $J_{2,3} = 15.5$ Hz, H-2 & 2′), 7.11 (2H, dq, $J_{1,2} = 6.95$ Hz, $J_{2,3} = 15.5$ Hz, H-3 & 3′) and 9.81 (br, 2H, CON*H*–N*H*CO). ^{13}C NMR spectral data ($CDCl_3$): δ 18.95 (q, C-4 & 4′), 123.02 (d, C-3 & 3′), 148.41 (d, C-2 & 2′), 172 (s, C-1 & 1′). (+)-ESIMS *m*/*z* at 169 $[M+H]^+$ and 191 $[M+Na]^+$. Elemental analysis: found C, 57.08; H, 7.15; N, 16.62. $(C_4H_6NO)_2$ requires C, 57.13; H, 7.19; N, 16.66%.

3.4. Evaluation of anti-genotoxic effect of (E)-N′-[(E)-2-butenoyl]-2-butenoylhydrazide **2**

Anti-genotoxic effect of hydrazide compound **2** was tested by analyzing H_2O_2 induced chromosomal aberrations in root tip cells of

Allium cepa in the presence and absence of hydrazide compound **2**. Five commercial equal size onion bulbs of 3–4 g were used. They were carefully unscaled, placed on top of test tube filled with tap water and allowed to germinate in the dark at 22 °C for 48 h. After 48 h, two unhealthy onion with the most poorly growing roots (>1 cm) were removed and the other healthy onion bulbs in water were treated with 0.7% H_2O_2 for 1 h. After the H_2O_2 treatment, roots were washed for 1 h and onion bulbs were treated with different concentrations (1.9–250 ppm; prepared in distilled water) of hydrazide compound **2** for 24 h. Three onion bulbs were used in each treatment.

H_2O_2 in the concentration was used as positive control for 1 h and tap water as negative control. After the completion of treatment (72 h), the root tips from each onion bulbs were fixed in Farmer's fluid (glacial acetic acid:ethyl alcohol, 1:3) for 24 h, transferred to 70% ethyl alcohol and stored at 4 °C.

For chromosomal analysis, squash preparation was made as follows: the root tips were hydrolyzed in 1 N HCl at 60 °C for 1 min and transferred to a watch glass containing 1 N HCl and aceto-orcein (1:9). They were then heated intermittently for 3–5 min. The tip of the root was cut, placed on glass slid in a drop of 45% acetic acid, covered with cover slip, squashed by tapping, sealed with DPX and observed under microscope. The cells were scored at different stage of mitotic cycle. Different types of chromosomal aberration were noticed at randomly picked 3 zones. Three slides were examined per onion in each group.

3.5. Statistical analysis

The results are expressed as mean ± SEM. Statistical analysis was performed using SPSS v 10 and significance of difference between controls and treated groups were determined using Student's *t*-test at $P \leq 0.05$.

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