

How To Elicit The Genotoxic Effect Of Aqueous Extract Of Ecballium elaterium Using Micronucleus Assay And DNA Single Strand Break Techniques

I Muhammad Said Shabbar

Citation

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Abstract

Medicinal green plants are generally containing mutagenic and carcinogenic substances, but there is little information about the biological activities of herbal medicine.[1] Ecballium elaterium (L.) ("squirting cucumber" or "donkey's green") is a perennial plant from the family Cucurbitaceae, a mediterranean medicinal plant that has been investigated for its several pharmacological properties.[2,3,4,5,6,7]

Dioscorides applied it to the medicine of which we are treating. [8, 9] Herbal medicine recommends it for the treatment of chronic sinusitis or rhinosinusitis [7, 10,11,12] It is also recommended for liver cirrhosis as well as other conditions thought to be inflammatory in nature including rheumatism and infections [13] Ecballium elaterium roots were used as analgesic and in treatment of hemorrhoids; fruits in sinusitis, jaundice, nocturia, lumbago and otalgia [7, 11, 14, 15]

THE REVIEW

A recent study conducted was designed to investigate the anti-inflammatory effect of aqueous extract of Ecballium elaterium, which is topically applied for treatment of rhinosinusitis as a traditional folk medicine in Turkey. Therapeutic potential of E. elaterium as an anti-inflammatory agent was examined by measurement of nitric oxide metabolites in a rabbit model of rhinosinusitis. The results showed that both activity of nitric oxide synthase enzyme and concentration of nitric oxide metabolites were significantly reduced by topical administration of E. elaterium extract in therapy group as compared to the control. Thus, the data suggest E. elaterium extract may have the potential to be used as anti-inflammation agent, and can be used in the treatment of rhinosinusitis diseases.[12]

A study has been made taking a series of 13 patients who were exposed to the juice of Ecballium elaterium in its natural form. In 3 patients, exposure was intranasal for the treatment of sinusitis or liver cirrhosis. In 3 other cases, children ingested the fruit unwittingly. In 6 patients, exposure was ocular and, in one, dermal. Within minutes of exposure, the patients exhibited irritation of mucous membranes at various degrees of severity manifested as edema of pharynx, dyspnea, drooling, dysphagia, vomiting, conjunctivitis, corneal edema, and erosion, depending on the

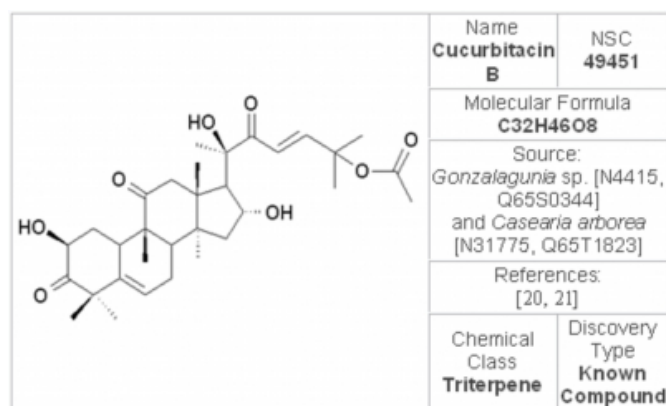
route of the exposure. Recovery began within several to 24 hours after administration of oxygen, steroids, antihistamines, and beta-2-agonists. Ocular exposures responded to topical steroid and antibiotic eyedrops within few days. The toddler with the dermal exposure remained asymptomatic. The conclusion of this study was that exposure to the juice of Ecballium elaterium, mainly in its undiluted form, may cause irritation of mucous membranes, supposedly of inflammatory nature. Patients exposed orally or intranasally should be closely followed for upper airway obstruction. Patients exposed ocularly should have their eyes promptly irrigated to prevent corneal and conjunctival injury [16] Another study [17] tested the in vitro pollen germination on Ecballium elaterium (L.) A. Rich. (Cucurbitaceae) and other 6 different plant species in basic medium. Sucrose (10%) and boric acid (0.01%) was used as germination medium and germination occurred in Petri dishes. The percentage of in vitro germination of pollen tubes among the species was found to be quite different. Percentage of germination for Ecballium elaterium was 5%.

Ecballium elaterium fruit juice initial characterization using SDS-PAGE indicated the presence of 20 proteins with molecular weights ranging from 13 to 103 KDa. Heat treatment of the juice led to the loss of 3 proteins and appearance of another. Moreover, 3 proteins stained

positively with periodic Schiff stain (PAS) for carbohydrate detection. In male Sprague Dawley albino rats, the LD₅₀ was estimated to be 2.5 mg/kg body weight, when injected intravenously. The crude juice resulted in agglutination of red blood cells from various species. This effect was species-specific, with rabbit's RBCs being the most affected and those of sheep the least sensitive. Although the crude juice, at concentrations equal or greater than 0.5 mg/ml, induced coagulation of the human plasma, it was found to delay prothrombin time. Heat treatment augmented dermonecrotizing effects of the crude juice in rats and Balb/c mice. Furthermore, heating increased the killing activity in human blood mononuclear cells [18]. The extracts of *Ecballium elaterium* used for various disorders in Anatolia were investigated in vitro for its neutrophil chemotactic and intracellular killing activities by Boyden migration chamber and nitroblue tetrazolium (NBT) dye reduction tests, respectively. NBT results indicated that the extracts of *Ecballium elaterium* have the ability to reduce active radicals suggesting that the extract is also valuable in the medical treatment of some diseases associated with free radical damage [19]. Earlier study [7] have identified the active principle of fruit juice as cucurbitacin B shown in figure (1), which possesses in vivo potent anti-inflammatory activity against increased vascular permeability induced by acetic acid in mice.

Figure 1

Figure 1: Cucurbitacin B structure



Cucurbitacin E (CuE) extracted from *Ecballium elaterium*, like other cucurbitacins, is a compound class of highly oxygenated and structurally diverse tetracyclic triterpenes. They have been mainly reported from Cucurbitaceae but are also known from other plant families [22, 23]. Cucurbitacins taste bitter and show a broad range of bioactivities [24, 25]. CuE shown in figure (2) is a potential anti-neoplastic agent.

A study has been made to verify this; it investigated the in vitro effects of CuE on phytohaemagglutinin (PHA)-activated and unstimulated lymphocytes. CuE did not produce any significant cytotoxic effects on the two models. On the contrary, it had a stimulatory effect, and was capable of inducing and maintaining high proliferation rates in lymphocytes. The stimulatory effect of CuE was concentration-dependent with a median stimulatory concentration (SC₅₀) of 1.166 mM and reaching maximal effect at a concentration of 10–20 mM. The stimulatory effect of CuE

was about 25% lower than that of PHA, but a combination of CuE and PHA had an additive effect producing a greater response than that induced by the two substances used separately. Agarose gel electrophoresis for DNA fragmentation failed to show any significant apoptotic activity in the cells after 48 hrs exposure to CuE [26]

The immunomodulatory effect of CuE, was tested in peripheral human lymphocytes. These lymphocytes were co-cultured with cancer cells and an interesting lymphocyte-mediated cytotoxicity was observed. [27] A Spectral Assignments and Reference Data has been done by 2D NMR-derived ¹H and ¹³C NMR signal assignments of six structurally closely related cucurbitacin derivatives. The investigated aglyca CuE and I were isolated from *Ecballium elaterium* L whereas the 2-O-β-D-glucopyranosylcucurbitacins I, J, K, and L were obtained from *Citrullus colocynthis* (L.) Schrader [28], another ¹H and ¹³C NMR signal assignments derived from 2D NMR experiment based correlations are presented for 22-deoxocucurbitacin D and cucurbitacin D shown in figure (3) which were isolated from *Ecballium elaterium* L. (Cucurbitaceae) [29]

Figure 2

Figure 2: Cucurbitacin E structure.

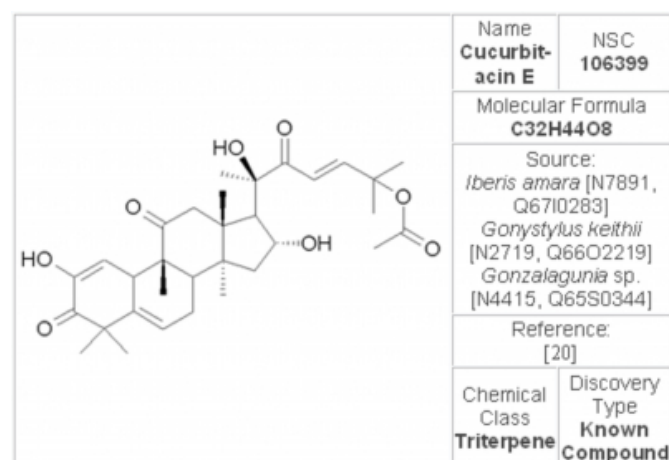
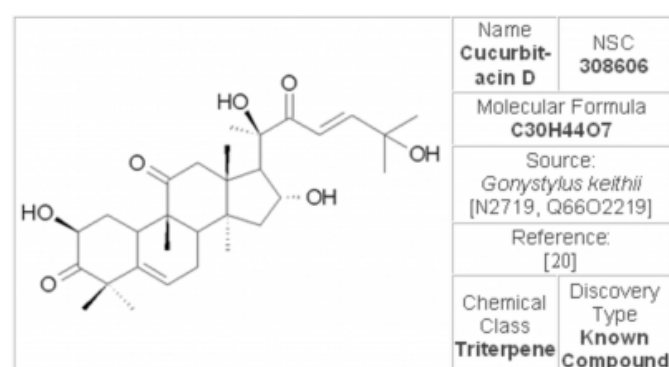


Figure 3

Figure 3: Cucurbitacin D structure



The effect of cucurbitacin of *Ecballium elaterium* extract on the formation of mRNA coding for laccase was examined in cultures of *Botrytis cinerea* grown with inducers of laccase formation, in the presence or absence of the inhibitory compounds. RNA was isolated from cultures and probed with specific DNA probes for laccase. As an internal control, the RNA was probed for *Botrytis* β -tubulin mRNA. From an analysis of the results of the Gonen et al., 1996 study, it is clear *Ecballium* extract specifically repress the amount of mRNA coding for laccase. This could account for the previously observed repression of laccase formation by cucurbitacins [30]

The *Ecballium elaterium* trypsin inhibitor II (EETI-II), a member of the squash family of protease inhibitors, is composed of 28 amino acid residues and is a potent inhibitor of trypsin. Its compact structure is defined by a triple-stranded anti-parallel β -sheet, which is held together by three intramolecular disulfide bonds forming a cystine knot. In

order to explore the potential of the EETI-II peptide to serve as a structural scaffold for the presentation of randomized oligopeptides, [31] constructed two EETI-II derivatives, where the six-residue inhibitor loop was replaced by a 13-residue epitope of Sendai virus L-protein and by a 17-residue epitope from human bone Gla-protein. EETI-II and derived variants were produced via fusion to maltose binding protein MalE. By secretion of the fusion into the periplasmic space, fully oxidized and correctly folded EETI-II was obtained in high yield. EETI-II and derived variants could be presented on the *Escherichia coli* outer membrane by fusion to truncated Lpp'-OmpA' which comprises the first nine residues of mature lipoprotein plus the membrane spanning β -strand from residues 46-66 of OmpA protein. Gene expression was under control of the strong and tightly regulated tetA promoter/operator. Cell viability was found to be drastically reduced by high level expression of Lpp'-OmpA'-EETI-II fusion protein. To restore cell viability, net accumulation of fusion protein in the outer membrane was reduced to a tolerable level by introduction of an amber codon at position 9 of the lpp' sequence and utilizing an amber suppressor strain as expression host. Cells expressing EETI-II variants containing an epitope were shown to be surface-labeled with the respective monoclonal antibody by indirect immuno-fluorescence corroborating the cell surface exposure of the epitope sequences embedded in the EETI-II cystine knot scaffold. Cells displaying a particular epitope sequence could be enriched 10^7 -fold by combining magnetic cell sorting with fluorescence-activated cell sorting. These results demonstrate that *E. coli* cell surface display of conformationally constrained peptides tethered to the EETI-II cystine knot scaffold has the potential to become an effective technique for the rapid isolation of small peptide molecules from combinatorial libraries that bind with high affinity to acceptor molecules [31] Another test was done on EETI-II indicated that EETI-II is one of the strongest inhibitors known for trypsin. The eight independent molecules of EETI-II in the crystal structure reported here provide a good opportunity to test the hypothesis that this small cystine-knot protein (knottin) is sufficiently rigid to be used as a molecular scaffold for protein-engineering purposes. To extend this test, the structures of two complexes of EETI-II with trypsin have also been determined, one carrying a four-amino-acid mutation of EETI-II. The remarkable similarity of these structures confirms the rigidity of the molecular framework and hence its suitability as a molecular scaffold. [32]

MICRONUCLEUS ASSAY

The micronucleus assay is a cytogenetic test commonly used in various biological systems for monitoring environmental genotoxicity, both in vitro and in vivo. The description of in vivo micronucleus assay is the primary focus of this study.

The in vivo test is especially relevant to assessing genotoxicity hazard in that it allows considerations of factors of in vivo metabolism [33, 34]. A micronucleus is a small extranucleus separated from the main one, generated during cellular division by late chromosomes or by chromosome fragments. Micronucleus has been applied to peripheral blood lymphocytes and lesser in epithelial cells [35, 36]. In this method, the animals were treated with test agent by appropriate route, peripheral blood cells were collected after treatment, smear slides were prepared, studied, coded and analyzed for the toxicity and micronucleated cell frequency [34, 37].

DETECTION OF SINGLE STRAND BREAKS

DNA strand breaks can be categorized as either single-strand break (SSB) or double-strand break (DSB). SSB normally represent repairable lesions, because the opposite strand holds both ends close together. In contrast, however, double strand breaks are usually considered to be lethal, because they are not easy repairable. Anyhow, detection of DNA strand breaks, either directly induced, or transiently induced during repair processes can be considered as a helpful tool to test the genotoxic properties of chemicals and particles [38].

References

1. Basaran, A.; Ahmet, Yu; Tian-Wei, P.; Michael J. and Anderson, D. An investigation of some Turkish herbal medicines in *Salmonella typhimurium* and in the COMET assay in human lymphocytes. *Teratog Carcinog Mutagen.* 1996;16 (2): 125-138.
2. Baytop, T. Therapy with medicinal plants in Turkey (Past and Present). Nobel Tip Publications, Istanbul. pp. 348-349 1999.
3. Wiersema, J.H. and Leon, B. *World Economic Plants*. CRC Press. P. 194 1999.
4. Basaran, A.; Basaran, N. and Baser, K.H. Chromosome aberrations induced by aflatoxin B1 in rat bone marrow cells in vivo and their suppression by *Ecballium elaterium*. *Fitoterapia.* 1993; 64 (4): 310-313.
5. Elayan, H.H.; Gharaibeh, M.N.; Zmeili, S.M. and Salhab, A.S. Effects of *Ecballium elaterium* juice on serum albumin concentration in male rats. *Intl J Crude Drug Res.* 1989; 27, 227-234.
6. Favel, A.; Mattras, H.; Coletti-Previero, M.A.; Zwilling, R.; Robinson, E.A. and Castro, B. Protease inhibitors from *Ecballium elaterium* seeds. *Int J Pept Protein Res.* 1989; 33, 202-208.
7. Yesilada, E.; Tanaka, S.; Sezik, E. and Tabata, M. Isolation of an anti-inflammatory principle from the juice of *Ecballium elaterium*. *J Nat Prod* 1988; 51, 504-508.
8. Culbreth, David M.R. *A Manual of Materia Medica and Pharmacology*, (7th edition), Lea & Febiger, Philadelphia, USA, p. 80 1927.
9. Wood, H.C.; Bache, F.; Remington, J.P. and Sadtler, S.P. *The Dispensary of the United States of America*, (Twentieth Edition) Lippincott company, Philadelphia and London, p. 2. 1918.
10. Plitmann, U.; Heyn, C.; Danin, A. and Shmida, A. *Pictorial Flora of Israel*. Massada, Tel Aviv. p. 344. 1983.
11. Lewis, W.H. *Medical Botany: Plants Affecting Man's Health*. New York: Wiley and Sons. 1977.
12. Uslu, C.; Karasen, R.M.; Sahin, F.; Taysi, S. and Akcay, F. Effect of aqueous extracts of *Ecballium elaterium* rich., in the rabbit model of rhinosinusitis. *Int J Pediatr Otorhinolaryngol.* 2006; 70(3):515-518.
13. Sezik, E. and Yesilada, E. Clinical effects of the fruit juice of *Ecballium elaterium* in the treatment of sinusitis [letter]. *J Toxicol Clin Toxicol.* 1995; 33 (4): 381-382.
14. Yesilada, E. *Biodiversity in Turkish Folk Medicine in "Biodiversity Biomolecular aspects of biodiversity and innovative utilization"* Ed. Sener, B. Kluwer Academic, Plenum Publishers, London. 2002.
15. RTECS (Registry of Toxic Effects of Chemical Substances). *Tomes CPS System*, Englewood, Colorado: Micromedex Inc. 39. 1999.
16. Raikhlin-Eisenkraft, B. and Bentur, Y. *Ecballium elaterium* (Squirting Cucumber)-Remedy or Poison?. *Clin. Toxicol.* 2000; 38 (3): 305-308.
17. Dane, F.; Olgun, G. and Dalgıç, Ö. In vitro pollen germination of some plant species in basic culture medium. *J. Cell Molec. Biol.* 2004; 3, 71-76.
18. Khalil, A.M. and Qaoud, K.M. Toxicity and Partial Characterization of *Ecballium elaterium* fruit juice. *Int. J. Pharmacog.* 1993; 31 (2): 135-141.
19. Başaran, A.A.; Ceritoğlu, I.; Ündeğer, Ü and Başaran, N. Immunomodulatory activities of some Turkish medicinal plants. *Phytother. Res.* 1997; 11, 609-611.
20. Fuller, R.W.; Cardellina, J.H., II; Cragg, G.M. and Boyd, M.R. Cucurbitacins: differential cytotoxicity, dereplication and first isolation from *Gonystylus keithii*. *J. Nat. Prod.* 1994; 57(10): 1442-1445.
21. Beutler, J.A.; McCall, K.L.; Herbert, K.; Herald, D.L.; Pettit, G.R.; Johnson, T.; Shoemaker, R.H. and Boyd, M.R. Novel cytotoxic diterpenes from *Casaria arborea*. *J. Nat. Prod.* 2000; 63(5): 657-661.
22. Guha, J and Sen, SP. The cucurbitacins-a review. *Plant Biochem J.* 1975; 2, 12-28.
23. Lavie, D and Glotter, E. The cucurbitacins, a group of tetracyclic triterpenes. *Fortschritte Chemie organischer Naturstoffe.* 1971; 29, 308-362.
24. Miro, M. Cucurbitacins and their pharmacological effects. *Phytother. Res.* 1995; 9, 159-168.
25. Setzer, W.N. and Setzer, M.C. Plant-derived triterpenoids as potential antineoplastic agents. *Min. Rev. Med. Chem.* 2003; 3, 540-556.
26. Attard, E.; Cuschieri, A.; Scicluna-Spiteri, A. and Brincat, M.P. The Effects of Cucurbitacin E on two lymphocyte models. *Pharmaceut. Biol.* 2004; 42 (2): 170-175.
27. Attard, E.; Brincat, M.P. and Cuschieri, A. Immunomodulatory activity of cucurbitacin E isolated from *Ecballium elaterium*. *Fitoterapia* 2005; 76, 439-441.
28. Seger, C.; Sturm, S.; Mair, M.E.; Ellmerer, E.P. and Stuppner, H. Spectral assignments and reference data. *Magn. Reson. Chem.* 2005; 43, 489-491.
29. Seger, C.; Sturm, S. and Haslinger, E. NMR Signal assignment of 22-Deoxocucurbitacin D and Cucurbitacin D

from *Ecballium elaterium* L. (Cucurbitaceae). Monatshefte für Chemie. 2005; 136, 1645-1649

30. Gonen, L.; Viterbo, A.; Cantone, F.; Staples, R.C. and Mayer, A.M. Effect of cucurbitacins on mRNA coding for Laccase in *Botrytis cinerea*. Phytochemistry. 1996; 42 (2): 321-324

31. Christmann, A.; Walter, K.; Wentzel, Alexander; Krätzner, R. and Kolmar, H. The cystine knot of a squash-type protease inhibitor as a structural scaffold for *Escherichia coli* cell surface display of conformationally constrained peptides. Protein Eng. 1999; 12 (9): 797-806

32. Krätzner, R.; Debreczeni, J.E.; Pape, T.; Schneider, T.R.; Wentzel, A.; Kolmar, H.; Sheldrick, G.M. and Usona, I. Structure of *Ecballium elaterium* trypsin inhibitor II (EETI-II): a rigid molecular scaffold. Acta Cryst. 2005; D61, 1255-1262

33. Mersch, J. and Beauvias, M.N. The micronucleus assay in the zebra mussel, *Dreissena polymorpha*, in situ monitor genotoxicity in freshwater environments. Mutation Res. 1997; 393 (1-2): 141-149

34. Krishna, G. and Hayashi, M. In vivo rodent micronucleus assays: protocol, conduct, and data interpretation. Mutation Res. 2000; 455 (1-2): 155-166

35. Krisch-Volders, M. and Fenech, M. Inclusion of the micronuclei in non-divided mononuclear lymphocytes and necrosis/apoptosis may provide a more comprehensive cytokinesis block micronucleus assay for biomonitoring purposes. Mutagenesis. 2001; 16 (1): 51-58.

36. Ramierz, A. and Saldanha, P.H. Micronucleus investigation of alcoholic patients with oral carcinoma. Genet. Mol. Res. 2002; 1 (3): 246-260.

37. Schmid, W. The micronucleus test. Mutation Res. 1975; 31 (1): 9-15.

38. Shi, T. Electron paramagnetic resonance as a method to monitor the intrinsic oxidant activity of ambient Particulate Matter (PM), design, validation and application of the method in environmental settings. Ph.D thesis. Faculty of medicine of the Heinrich-Heine-Universität, Düsseldorf, Germany. 2003

Author Information

Ibrahim Muhammad Said Shabbar, B.Sc.

Post Graduate Student, Yarmouk University