# *VERBENA OFFICINALIS* ESSENTIAL OIL AND CITRAL AS APOPTOTIC INDUCTORS IN LEUKOCYTES OF HEALTHY SUBJECTS AND CHRONIC MYELOID LEUKEMIC PATIENTS

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## Summary

*Verbena officinalis* L. (vervain, Verbenaceae) has been used as folk medicine for thousands of years and in some countries has been reported as anticancer agent. Despite its widespread use, the mechanisms of pharmacological actions of the herb are not still cleared. Vervain essential oil, which has several traditional medicinal uses, is almost completely constituted of isoprenoid compounds. Purpose of this study was to evaluate the pro-apoptotic activity of vervain essential oil and of its main compound, citral, on neutrophil granulocytes collected from normal blood donors and chronic myeloid leukemic patients (CML). Vervain essential oil induced a significant apoptosis (vs control) in granulocytes from both healthy donors and chronic myeloid leukemia patients: percentage of apoptotic cells was significantly greater in CML patients.

**Keywords**: *Verbena officinalis*, citral, apoptosis, chronic myeloid leukemia

## Introduction

Numerous bioactive constituents have been hypothesized to act as cancer-preventing agents by inhibiting the activation of pro-carcinogens, enhancing the detoxification of carcinogens, or impeding the progression of carcinogenesis (1-2). One of the most promising mechanism in order to block the cancer progression is a selective induction of apoptosis in neoplastic cells. Preliminary evidence indicates that isoprenoids, a broad class of mevalonate-derived phytochemicals which are ubiquitous in the plant kingdom, may suppress, with great potency, the proliferation of tumor cells as human breast adenocarcinoma (MCF7), human leukemia (HL-60) and human colon adenocarcinoma (CaCo 2) (3-4).
*Verbena officinalis* L. (Verbenaceae), commonly known as vervain, is a medicinal plant which grows wild everywhere. The herb has been used as folk medicine for thousands of years and in some countries has been reported as anticancer agent. The plant has been approved as herbal medicine and dietary supplement by many regulatory acts in many countries (5). Despite its widespread use, the mechanisms of pharmacological actions of the herb are still unclear (5). *Verbena officinalis* essential oil which has several traditional medicinal uses is almost completely constituted of isoprenoid compounds. Purpose of this study was to evaluate the pro-apoptotic activity of vervain essential oil and of its main compound, citral, on neutrophil granulocytes collected from normal blood donors and chronic myeloid leukemic patients (CML).

## Methods

The essential oil of vervain was purchased by A.C.E.F. spa (Fiorenzuola d´Arda, Italy). The chemical composition of the oil (Table 1) was obtained by Gas-Chromatography and GC-Mass spectrometry methods, as previously reported (6). Citral was purchased by Sigma-Aldrich Co, Milan, Italy.

Table 1 – Percentage composition of *Verbena officinalis* essential oil.

| | |
|---|---|
| α-Pinene | 0,2 |
| Sabinene | 0,5 |
| β-Pinene | T |
| Hepten-2-one | 0,2 |
| α-Terpinene | T |
| *o*-Cymene | 0,1 |
| Limonene | 3,3 |
| 1,8-Cineole | 0,4 |
| β- Phellandrene | 0,6 |
| *cis*-Ocimene | T |
| *trans*-Ocimene | 0,2 |
| γ-Terpinene | 0,1 |
| Terpinolene | T |
| Linalol | 0,1 |
| *trans*-Pinocarveol | T |
| *trans*-Pinocamphone | T |
| Pinocarvone | T |
| Borneol | 0,1 |
| *cis*- Pinocamphone | 0,1 |
| Terpinen-4-ol | 0,2 |
| *p*-Cymen-8-ol | T |
| α-Terpineol | 0,2 |
| Isobornyl formate | 45,4 |
| Citral | 45,5 |
| Isobornyl acetate | T |
| Bornyl acetate | T |
| Anethole | 0,2 |
| α-Copaene | 0,2 |
| Isoledene | 0,1 |
| β-Elemene | 0,2 |
| Eugenol methyl ester | T |
| Methyl eugenol | T |
| Longifolene | T |
| β-Caryophyllene | 0,9 |
| β-Cedrene | 0,4 |
| α-Humulene | 0,2 |
| *allo*-Aromadendrene | 0,1 |
| γ-Gurjunene | T |
| *cis*-Muurola-4(14),5-diene | 0,2 |
| Bicyclogermacrene | 0,1 |
| α-7-epi-Selinene | 0,1 |

Whole blood samples (3 mL in EDTA) were collected from 4 patients of the "AA.OO. "San Giovanni di Dio e Ruggi d'Aragona", 2 men and 2 women, 55-75 years old, affected by Chronic Myeloid Leukemia and from blood donors, healthy subjects, 2 men and 2 women, aging from 35 to 58, which were member of the staff of the Department of Pharmaceutical Sciences at the University of Salerno. Informed consent on the purpose and the modality of the study was obtained from all subjects.

Leucocytes isolation was achieved through erythrocytic lysis with $NH_4Cl$ (Autolyse $^{PLUS}$ Bio Source) and centrifugation at 1200 rpm for 7 minutes. The treated leukocytic pellets were suspended in a mixture of 90 µL of PBS (Biomérieux) and 0,1 µL of vervain essential oil, 0,1 µL of acetone and 9,8 µL of distilled water (to obtain vervain essential oil diluted 1:100) or 10 µL of pure citral at the concentration of 1,9 mM. Controls were performed treating both with only 100 µL of PBS and with a mixture of 90 µL of PBS plus 10 µL of acetone. The proapoptotic effect of the compounds was evaluated after three different times of incubation (6, 12, 24 hours in dark condition), by adding to non-treated and treated leukocytes annexin V 5µl and propidium iodide 5 µl [Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen)] in flow cytometry (FACSCalibur Becton Dickinson). The software to acquire and elaborate data was CELLQuest$^{TM}$, version 3.3 for Mac OS, Becton Dickinson.

Statistical analysis was done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Continuous variables are expressed as percentage mean and $\chi$ square test was used to compare groups.

## Results

In our study the control samples, in the three different incubation times, didn't showed apoptosis, but only necrotic cellular elements. Instead, the cytometric analysis in healthy and pathological cells treated with *Verbena officinalis* essential oil showed apoptotic elements (vs controls). Controls did not showed statistical difference.

In particular vervain essential oil induced a significant apoptosis (vs control) in granulocytes from both healthy donors and chronic myeloid leukemia patients. Percentage of apoptotic cells was greater in CML patients (76% after 6h of incubation) than in healthy subjects (56%, after 6 h of incubation) (Figures 1 and 2). Non treated granulocytes were necrotic in both healthy subject and in CML patients (Figures 1 and 2).

Figure 1: Apoptosis and necrosis in non treated granulocytes (control) or treated with vervain essential oil from normal blood donors.

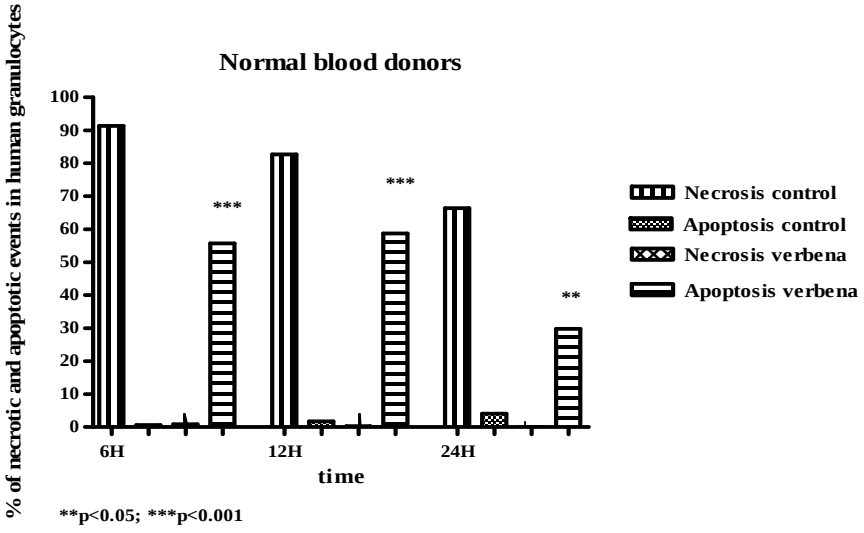
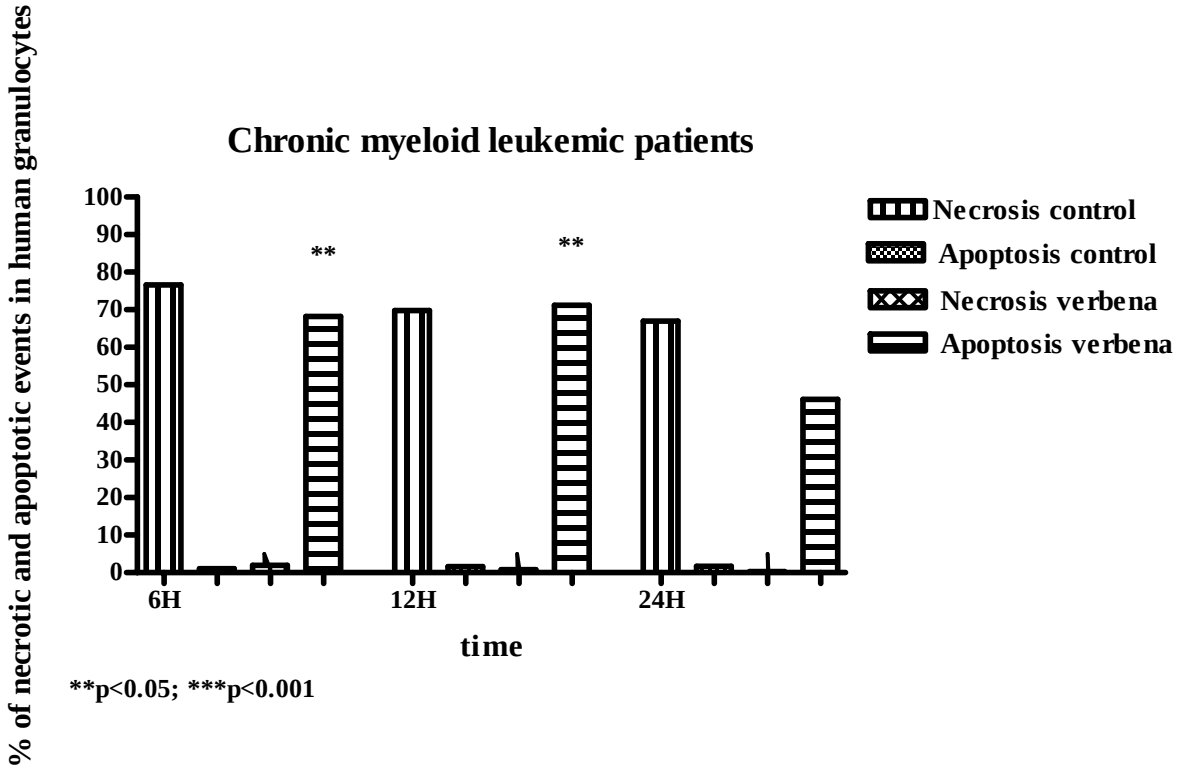


Figure 2: Apoptosis and necrosis in non-treated granulocytes (control) and treated with vervain essential oil from chronic myeloid leukemic patients.




Citral had a strong pro-apoptotic activity at the 1,9 mM concentration, both in healthy subjects (figure 3) and in CML patients (figure 4). CML granulocytes are more sensitive to citral than granulocytes of donors.

Figure 3: Apoptosis and necrosis in non-treated granulocytes (control) and treated with citral 1,9 mM, from normal blood donors.

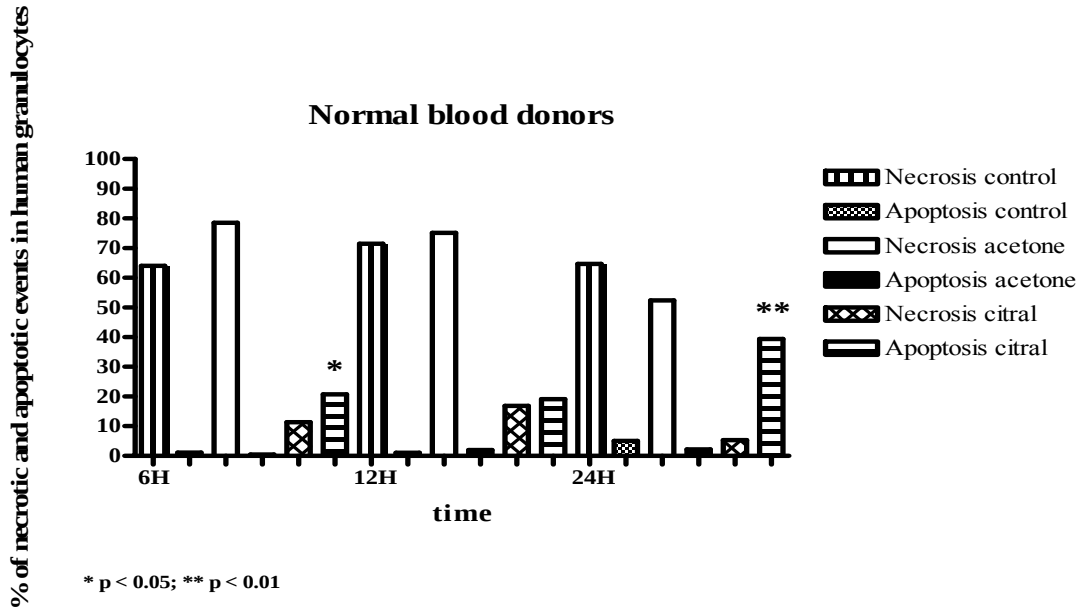
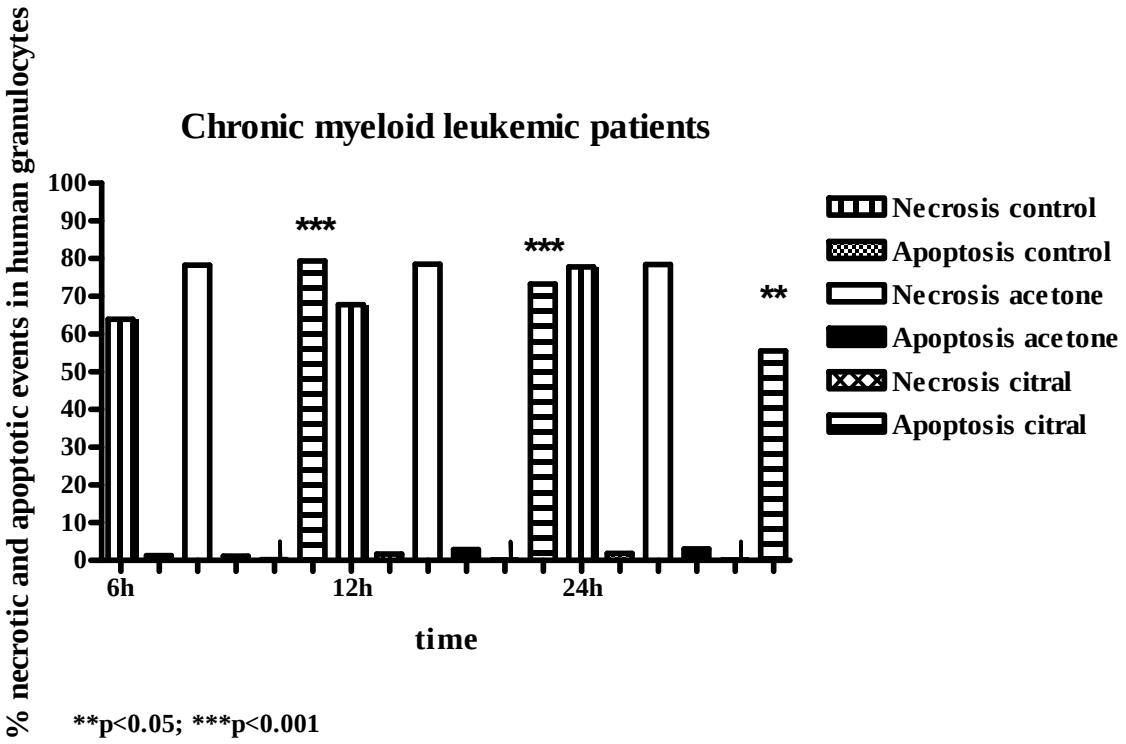


Figure 4: Apoptosis and necrosis in non-treated granulocytes (control) and treated with citral 1,9 mM from chronic myeloid leukemic patients.




## Conclusions

The obtained data are in accordance with other studies that evaluated the effect on cell cycle and apoptosis of some essential oil components in cancer cellular lines (7-8).
The molecular mechanism at the basis of this process is still unclear. The apoptotic effect which is induced by *Verbena officinalis* essential oil and citral could be related to activation of the caspase 3 (9).
The different proapoptotic effect observed in the two examinated subjects groups (blood donors and CML patients) can find a possible explanation in the fact that CML patients present a different clinic condition in comparison to normal blood donors and their neutrophil granulocytes would be more susceptible to the action of the drug.
Our data agree with literature that indicates natural compounds as lead structure to develope new therapeutic agents**.**

## References

1) Hursting SD, Fischer SM, Wargovich MJ, Digiovanni J. Nutritional modulation of the carcinogenesis process. In: Heber D, Blackburn G eds. Nutritional Oncology. San Diego, CA: Academic Press, 1999: 91–104.
2) Wattenberg LW. Inhibition of carcinogenesis by minor dietary constituents. Cancer Res 1992; 52: 2085s–2091s.
3) Mo H, Elson CE. Apoptosis and cell-cycle arrest in human and murine tumor cells are initiated by isoprenoids. J Nutr 1999; 129: 804-813.

4) Tatman D, Mo H. Volatile isoprenoid constituents of fruits, vegetables and herbs cumulatively suppress the proliferation of murine B16 melanoma and human HL-60 leukemia cells. Cancer Lett 2002; 175: 129-139.
5) Sau-Wan L, Man-Shan Y, Wai-Hung Y, Chuen-Chung Chan R. Novel neuroprotective effects of the aqueous extracts from *Verbena officinalis* Linn. Neuropharmacology 2006; 50: 641-650.
6) De Feo V, Bruno M, Napolitano F, Tahiri B, Senatore F. Chemical Composition and Antibacterial Activity of Essential Oils from *Thymus spinulosus* Ten. (Lamiaceae). J Agric Food Chem 2003; 51: 3849-3853.
7) Carnesecchi S, Schneider Y, Ceraline J, Duranton B, Gosse F, Seiler N, Raul F. Geraniol, a component of plant essential oils inhibits growth and polyamine biosynthesis in human colon cancer cells. J Pharmacol Exp Ther 2001; 298(1): 197-200.
8) Bardon S, Picard K, Martel P. Monoterpenes inhibit cell growth, cell cycle progression and cyclin D1 gene expression in human breast cancer cell lines. Nutr Cancer 1998; 32(1): 1-7.
9) Dudai N, Weinstein Y, Krup M, Rabinski T, Ofir R. Citral is a new inducer of caspase-3 in tumor cell lines. Planta Med 2005; 71(5): 484-488.