# Antioxidative, Antiproliferative and Biochemical Effects in HepG2 Cells of a Homeopathic Remedy and its Constituent Plant Tinctures Tested Separately or in Combination


Keywords: Antioxidants, cell proliferation, glutathione conjugation, hepatoprotection, homeopathy, plant tinctures

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## SUMMARY

Hepeel® is a homeopathic remedy commonly used to treat primary and secondary functional disorders of the liver. It consists of highly diluted extracts from the following plants: Chelidonium from Chelidonium majus, L., Carduus marianus from Silybum marianum, L., Veratrum from Veratrum album L., Colocynthis from Citrullus colocynthis L., Lycopodium from Lycopodium clavatum L., Nux moschata from Myristica fragans, Houtt, and China from Cinchona pubescens, Vahl. The antioxidative, antiproliferative and biochemical effects in HepG2 hepatoblastoma cells of serial dilutions of these plant tinctures were tested, either separately or in various combinations. Upon damage of the cells with tert-butyl hydroperoxide, Carduus marianus, China and Nux moschata, in decreasing order, showed the strongest antioxidative effects. Greater than 95% inhibition of total production of malondialdehyde was reached with these three tinctures at dilutions of D4. The complete combination of the tinctures (COMB) realized in the homeopathic remedy showed an effect corresponding to the combined effects of the individual tinctures. The antiproliferative influence on the incorporation of 3H-thymidine into DNA in normal HepG2 cells was significant ($p<0.01$) but relatively weak, and decreased in the order Carduus marianus, Chelidonium, Colocynthis and Veratrum. At a dilution of D4 (4X)Colocynthis showed the strongest inhibition (13.5%). The effect of the combination of Colocynthis and Veratrum was markedly higher (22.3%) than that of the individual tinctures, but was not additive. With this combination, cell numbers were reduced. COMB had similar effects on proliferation and cell numbers, with the antiproliferative effect starting at a dilution of 1:40. The conjugation of 3,4-dichloronitrobenzene with glutathione was induced only by Carduus marianus and COMB, while all other tinctures were ineffective. Neither the individual tinctures, nor COMB showed cytotoxic effects in the dilutions tested. These results demonstrate that the complete combination (COMB) realized in the homeopathic remedy and its constituents exert specific antioxidative, antiproliferative and biochemical effects on HepG2 cells which all point to a potential hepatoprotective and tumouristatic action.

## 1. INTRODUCTION

Oxidative stress is a key factor in many human diseases [1,2]. Reactive oxygen species (ROS) have the potential to damage nucleic acids, proteins and biomembranes. When cellular defense mechanisms fail, severe dysfunction or cell death can result, events that are part of the respective pathogenic process. There is accumulating evidence that plant-derived antioxidants may reduce or prevent oxidative stress and have a beneficial influence on animal and human health [3,4]. For example, polyphenols, as present e.g. in red wine, are known to reduce the risk for arteriosclerosis [5] and fibrosis [4,6]. Besides prevention of cellular and organ pathogenesis, many plant constituents, especially flavonoids and certain alkaloids, support regenerating mechanisms and/or inhibit uncoordinated growth of cells as may occur during carcinogenesis [7,8]. For example, silibinin was recently shown to inhibit growth of human prostate carcinoma cells [9].

Furthermore, it is well known that plant-derived compounds may interfere with components of drug-metabolising pathways [10]. In particular, detoxifying mechanisms including glutathione (GSH)-dependent conjugation reactions are frequently enhanced, resulting in additional protection against radicals and electrophilic compounds [11,12].

Although such effects can be ascribed to specific plant components, our knowledge of the actions of compounds contained in different plant materials is rather limited. In addition, the mechanisms of actions are frequently not known and the relative contribution of components in plant remedies remain to be assessed under suitable experimental conditions in order to rank their possible effectiveness. Furthermore, these compounds may not always act independently, but may mutually modify the mode of action as well as the degree of their individual influence. Since these aspects are too complex to be analyzed in whole organisms, sophisticated cell culture assays have been developed that provide reliable and reproducible information on mechanistic details [13].

In the present study we have focused on the comparison of seven plant tinctures which constitute a homeopathic remedy. **This remedy is frequently used to stimulate liver function in acute cases and chronic diseases, e.g., cholangitis and cholecystitis.** We studied the effects of the separate tinctures (prepared according to the homeopathic pharmacopoeia) and various combinations thereof with respect to antioxidative and antiproliferative potential as well as possible influence on biochemical aspects of biotransformation in the human hepatoblastoma cell line HepG2. Special emphasis was placed on the question of whether the effects of these tinctures are modified by serial dilutions or by the combination of the individual tinctures as used in the commercial product.



## 2. MATERIALS AND METHODS

### 2.1. Materials

The composition of the homeopathic remedy Hepeel® is shown in Table 1. Basic tinctures prepared from seven different plants, according to procedures 3a and 4a of the homeopathic pharmacopoeia, were provided by Biologische Heilmittel Heel GmbH (Baden-Baden, Germany). Specifically, the following tinctures were used: a) "Chelidonium" prepared from Chelidonium majus, L. (Ch-B 007009, D2), b) "Carduus marianus" prepared from Silybum marianum, L. (Ch-B 007034, D2), c) "Veratrum" prepared from Veratrum album L. (Ch-B 007050, D3), d) "Colocynthis" prepared from Citrullus colocynthis L. (Ch-B 007058, D3), e) "Lycopodium" prepared from Lycopodium clavatum L. (Ch-B 007001, D3), f) "Nux moschata" prepared from Myristica fragrans, Houtt (Ch-B 007026, D3), and g) "China" prepared from Cinchona pubescens, Vahl (Ch-B 007018, D3). The combination remedy of the tinctures in the potencies given above (+ "Phosphorus", a D4 potency of yellow phosphor) henceforth termed COMB was also supplied by Biologische Heilmittel Heel GmbH. Radiolabelled $^{3}$H-thymidine was purchased from Amersham Buchler (Braunschweig, Germany). All other chemicals were from Boehringer (Mannheim, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma (Daisenhofen, Germany).

### 2.2. Cultivation of HepG2 cells

HepG2 hepatoblastoma cells were cultured in Dulbecco's MEM (1x) medium (Gibco, Eggenstein, Germany) supplemented with 2 mM glutamine, 10% foetal calf serum, 40 U/mL streptomycin and 50 U/mL penicillin as described elsewhere [14,15]. Cells were passaged every week at the time they reached confluency. Stocks were kept frozen in liquid nitrogen. Frozen cells were thawed, cultured for one week, and passaged at least once before use. Confluent HepG2 cell cultures were used for all experiments.

### 2.3. Preparation of culture media containing test material

One part of the basic tinctures to be tested was mixed with 9 parts (v/v) of serum-free Williams Medium E and gently shaken for 20 min at room temperature. This stock solution was subsequently diluted with Williams Medium E as indicated in figures and tables. Appropriate controls contained an equal amount of ethanol to the respective test solution.

### 2.4. Determination of lipid peroxidation

Malondialdehyde (MDA) measurements were used to quantify lipid peroxidation [16]. Briefly, HepG2 cells were incubated together with t-butyl hydroperoxide (t-BHP; final concentration 1.5 mmol/l) and different concentrations of the test compounds for 60 min. After this time, the cells were washed with 0.9% NaCl and resuspended in 1 ml 50 mmol/l potassium phosphate buffer (pH 7.4) and homogenized by sonication for 10 s (15% of the maximum power, Sonopuls HD 2200, Bandelin electronic, Berlin, Germany). MDA was determined by thiobarbituric acid (TBA) assay [17,18].

Table 1: Constituent plant extract solutions of the homeopathic remedy

| Name | Source plant | Code | Dilution |
|---|---|---|---|
| Chelidonium | Chelidonium majus, L. | Ch-B 007009 | D2 |
| Carduus marianus | Silybum marianum, L. | Ch-B 007034 | D2 |
| Veratrum | Veratrum album L. | Ch-B 007050 | D3 |
| Colocynthis | Citrullus colocynthis L. | Ch-B 007058 | D3 |
| Lycopodium | Lycopodium clavatum L. | Ch-B 007001 | D3 |
| Nux moschata | Myristica fragans, Houtt | Ch-B 007026 | D3 |
| China | Cinchona pubescens, Vahl | Ch-B 007018 | D3 |

### 2.5. Determination of cell proliferation

Incorporation of $^{3}$H-thymidine (specific activity, 40 to 60 Ci/mmole) into DNA was used as an indicator of cell proliferation/entry into S-phase. Briefly, radiolabelled thymidine was added together with fresh culture medium to HepG2 cells. After an incubation period of 48 hrs, cells were washed four times in ice-cold trichloro acetic acid, dissolved in NaOH and processed for scintillation counting. Details are described elsewhere [19].

### 2.6. Determination of cytotoxicity and other analytical procedures

Cytotoxicity of the extracts and isolated compounds was determined using the MTT-assay (MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide) as described elsewhere [16]. Alternatively, the lactate dehydrogenase leakage assay was used as described [20]. Glutathione-S-transferase activity was determined using 1-chloro-2,4-dinitrobenzene according to Habig and Jacoby [21] with the modifications by Gebhardt *et al.* [22]. Protein was measured following the procedure of Lowry *et al.* [23].

### 2.7. Statistical evaluation

The data were evaluated statistically using Student's t-test. Data are given as the means ± standard deviations (SD) of three to four determinations.

## 3. RESULTS

The cytotoxic potential of all tinctures and combinations was determined using the MTT assay and the LDH leakage assay, in order to exclude unspecific or pleiotropic effects on the cells in the concentrations used. In addition, cultures were viewed microscopically for morphological changes. None of the tinctures and combinations tested showed any sign of a cytotoxic influence in any of the different test systems (not shown).

For determining the antioxidative capacity of the respective extracts, HepG2 cells were challenged with t-butyl hydroperoxide. This resulted in enhanced lipid peroxidation as evident from the cellular production of malondialdehyde (MDA) which increased approximately 2.5-fold within 1 h. As shown in Fig. 1 the complete combination corresponding to the homeopathic remedy (COMB) significantly reduced MDA production. Of the different tictures, Silybum marianum, Carduus marianus was the most effective (Fig. 2). Dilutions down to an effective concentration of 0.5-times D4 significantly reduced MDA production. At concentrations above D4, the reduction was greater than 70%. The effects of the different tinctures are compared in Table 2. Neither tinctures from Chelidonium, Veratrum, Colocynthis, and Lycopodium were effective, while China seemed equally potent as Carduus marianus and Nux moschata was slightly less effective. As apparent when comparing Figs. 1 and 2, the antioxidative influence of COMB, the combination of all extracts, very much resembled the effect of Carduus marianus. Obviously, China and Nux mochata contributed less to the effect, because of their higher dilution in COMB.

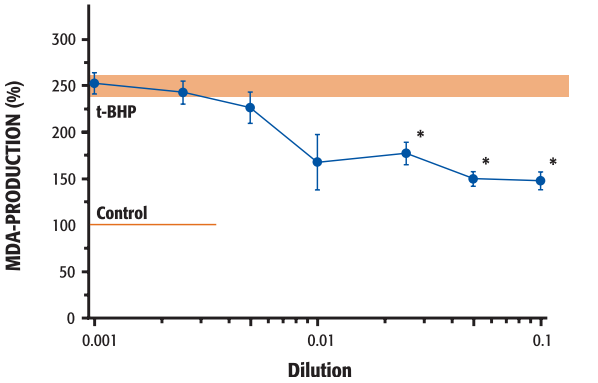


**Fig. 1:** Effect of different dilutions of the combination of all tinctures (COMB) on malondialdehyde production induced by t-butyl hydroperoxide. HepG2 cells were challenged by t-butyl hydroperoxide in the presence of different dilutions of COMB as described in Methods. Levels of malondialdehyde produced by t-butyl hydroperoxide alone ± SD are depicted by the shaded area; basal levels were set to 100% and are indicated by the horizontal line.
*, values are different from control, $p < 0.01$.

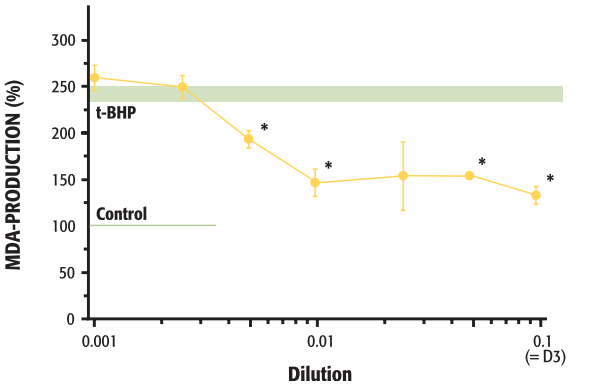


**Fig. 2:** Effect of different dilutions of Carduus marianus on malondialdehyde production induced by t-butyl hydroperoxide. HepG2 cells were challenged by t-butyl hydroperoxide in the presence of different dilutions of Carduus marianus as described in Methods. The starting concentration of Carduus marianus before dilution was D2. Levels of malondialdehyde produced by t-butyl hydroperoxide alone ± SD are depicted by the shaded area; basal levels were set to 100% and are indicated by the horizontal line.*, values are different from control, $p < 0.01$.

With respect to cell proliferation, a small degree of growth inhibition was observed with COMB (Table 2), where tinctures are already highly diluted. The lowest effective COMB dilution reducing DNA synthesis was 0.025. For COMB, reduction of cell number (approx. 8%) was also significant ($P < 0.05$). Of the constituent tinctures, a small, but significant ($p < 0.01$) reduction of $^{3}$H-thymidine-incorporation into DNA of HepG2 cells was found for Carduus marianus and three other tinctures (Table 2). The lowest concentrations exerting a significant effect on the control cultures were as low as D5 for Carduus marianus, 0.25-times D4 for Colocynthis, 0.5-times D4 for Veratrum and 0.25-times D3 for Chelidonium. These differences in potency between the extracts from different plants were also seen at the level of cell numbers (not shown), but was not statistically significant probably due to the short incubation period. The only exception was Colocynthis which, at D4, showed the strongest inhibition amounting to 13.5 ± 0.5% and 11.8 ± 2.7% for thymidine incorporation and cell number, respectively. The two tinctures Lycopodium and Nux moschata, appared not to influence DNA synthesis and proliferation, while China at D4 apparently showed a tendency for stimulation. This effect, however, was minor and rapidly disappeared on further dilution (Table 2).

**Table 2:** Comparison of the antioxidative and antiproliferative potency of different plant tinctures and of their complete combination COMB on HepG2 cells

| Material | Starting Potency | Reduction of MDA Production (% t-BHP) | | Inhibition of 3H-thymidine incorporation (% control) | |
|---|---|---|---|---|---|
| | | Dilution 1:10 | Dilution 1:20 | Dilution 1:10 | Dilution 1:20 |
| Chelidonium | D2 | 4.4 ± 5.7 | 0 ± 4.2 | 10.5 ± 2.5* | 8.1 ± 3.0* |
| | D3 | -1.1 ± 6.2 | -0.4 ± 4.0 | 7.3 ± 4.5* | 6.8 ± 1.7* |
| Carduus marianus | D2 | 75.7 ± 5.3* | 62.5 ± 0.9* | 5.0 ± 2.5 | 8.8 ± 2.4* |
| | D3 | 66.6 ± 4.8* | 34.8 ± 5.6* | 7.7 ± 3.6* | 8.5 ± 1.8* |
| Veratrum | D3 | 7.4 ± 6.6 | 4.3 ± 4.7 | 8.2 ± 3.5* | 9.8 ± 2.7* |
| Lycopodium | D3 | 5.7 ± 5.2 | 3.0 ± 4.4 | 3.9 ± 2.4 | -3.8 ± 7.4 |
| Colocynthis | D3 | 0 ± 7.9 | -0.5 ± 7.0 | 13.5 ± 0.5* | 7.9 ± 1.9* |
| Nux moschata | D3 | 27.1 ± 6.3* | 9.2 ± 4.2* | 2.5 ± 0.7 | 6.9 ± 3.2 |
| China | D3 | 53.5 ± 9.5* | 35.7 ± 5.2* | -12.3 ± 8.3 | -0.7 ± 1.5 |
| COMB | | 69.4 ± 5.2* | 67.3 ± 4.3* | 9.9 ± 1.3* | 7.0 ± 1.9* |

* Significantly different from respective controls ($p < 0.01$)



The capacity for glutathione conjugation was induced only by Carduus marianus (Fig. 3). At 0.5-times D3, conjugation of 3,4-dichloronitrobenzene (DCNB) increased by almost 25% within 24 hrs of exposure. None of the other tinctures influenced GSH conjugation of this compound (not shown). COMB caused an induction of DCNB conjugation (Fig. 4) closely resembling that shown in Fig. 3 indicating the influence of the Silybum marianum constituents in this mixture.

For all three functions the influence of normal dilutions of the different tinctures was compared with those of matching potencies. Within the limits of this approach (only comparison with 10-fold dilutions) identical results were obtained for both types of samples.

**Table 3:** Comparison of the antioxidative and antiproliferative potency of different combinations of plant tinctures on HepG2 cells

| Material | Starting Potency | Reduction of MDA Production (% total) | | Inhibition of 3H-thymidine incorporation (% control) | |
|---|---|---|---|---|---|
| | | Dilution 1:10 | 1:20 | Dilution 1:10 | 1:20 |
| **Antioxidative effects:** | | | | | |
| Mixture 1: | | 96.2 ± 3.1* | 77.8 ± 5.6* | n.d. | n.d. |
| Carduus marianus | D3 | | | | |
| Nux moschata | D3 | | | | |
| China | D3 | | | | |
| Mixture 2: | | 96.2 ± 3.1* | 77.8 ± 5.6* | n.d. | n.d. |
| Carduus marianus | D3 | | | | |
| Nux moschata | D3 | | | | |
| **Antiproliferative effects:** | | | | | |
| Mixture 1: | | n.d. | n.d. | 17.1 ± 3.0* | 15.4 ± 4.5* |
| Chelidonium | D2 | | | | |
| Carduus marianus | D3 | | | | |
| Veratrum | D3 | | | | |
| Colocynthis | D3 | | | | |
| Mixture 2: | | n.d. | n.d. | 16.4 ± 3.8* | 14.7 ± 2.7* |
| Chelidonium | D2 | | | | |
| Carduus marianus | D3 | | | | |
| Mixture 3: | | n.d. | n.d. | 22.3 ± 4.6* | 16.9 ± 5.2* |
| Veratrum | D3 | | | | |
| Colocynthis | D3 | | | | |

* Significantly different from respective controls ($p < 0.01$)

In order to obtain a comprehensive overview about the mode of interactions between different tinctures comprising COMB, an additional set of measurements was performed with specific combinations to elucidate the antioxidative and antiproliferative functions. The specific combinations for the most effective tinctures for each function are specified in Table 3. As seen in this table, the antioxidative influence appears to be additive. When two or three of the most effective tinctures were combined, this caused the inhibition of not only the t-BHP-induced, but also a major part of the basal MDA-production. In contrast, although the antiproliferative effect increased generally in combinations compared with the individual tinctures, the effect was usually less than the sum of the individual values (Table 2). Notably, however, the enhanced growth inhibition exerted by the combination was not only seen on the level of thymidine incorporation, but also showed itself in a matching reduction of cell numbers (not shown) to what was seen with COMB.

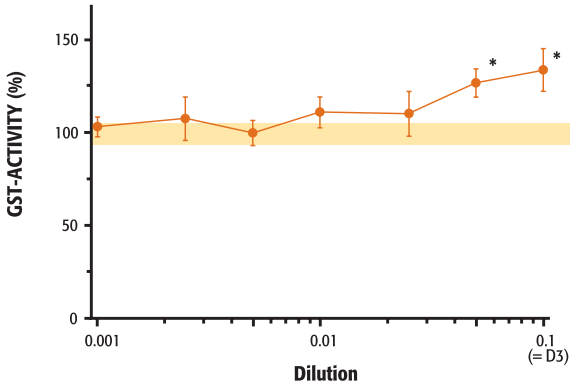


**Fig. 3:** Effect of different dilutions of Carduus marianus on induction of glutathione-S-transferase activity in HepG2 cells. Control levels were set to 100% and SD is indicated by the shaded area.*, values are different from control, $p < 0.01$.

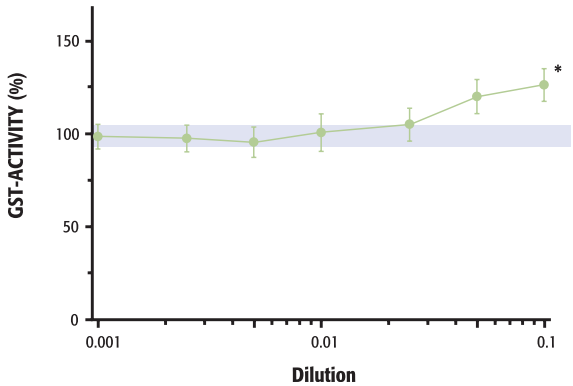


**Fig. 4:** Effect of different dilutions of the combination of all tinctures (COMB) on induction of glutathione-S-transferase activity in HepG2 cells. Control levels were set to 100% and SD is indicated by the shaded area.*, values are different from control, $p < 0.01$.

## 4. DISCUSSION

The results presented here show strong antioxidative and antiproliferative effects *in vitro* on the human hepatoblastoma cell line HepG2 of a homeopathic remedy as well as several of its constituent plant tinctures.

Depending on the test parameter the magnitude of the effects varied considerably between the different plant materials. The antioxidative effect decreased in the order Carduus marianus, China and Nux moschata. The antioxidative influence of COMB, the combination of all extracts, very much resembled the effect of Carduus marianus (cf. Fig. 2), which we attribute to the fact that China and Nux moschata are more diluted in the combination remedy (one potency higher) than Carduus marianus. For the antiproliferative effect, the sequence

with decreasing influence was Carduus marianus, Chelidonium, Colocynthis and Veratrum with Colocynthis showing the strongest inhibition of all at the lowest dilution. Only Lycopodium showed no effect on both of these functions. That the differences in potency between the extracts seen at the level of cell numbers was not statistically significant, was probably due to a too short incubation period.

The strong antioxidative influence of tinctures from Silybum marianum is in agreement with other reports, particularly those describing effects of the flavonolignans silibinin and silymarin using different antioxidant assay systems [24-26]. However, the almost equally potent antioxidative influence of China, the tincture from Cinchona pubescens is surprising, as only weak effects on free radical-mediated lipid peroxidation has been reported earlier for constituents such as quinidine [27,28]. Likewise, antioxidative properties of Nux moschata, the tincture from Myristica fragrans, have not been reported previously, although lignans and neolignans have been isolated from the bark of this plant [29] and may be responsible for such effects. On the other hand, it is interesting that Lycopodium did not affect t-BHP-induced MDA production, although one of its alkaloid constituent, huperzine A, has been shown to inhibit ROS production in cortical neurons of the rat [30]. Whether this discrepancy is due to different concentrations of respective components or to a cell-selective effect of Lycopodium remains to be determined.

When the most active tinctures were combined, the antioxidative effect appeared to be additive, amounting to an almost complete suppression of MDA production, including the basal production. Similar effects have been found with artichoke extracts which exert strong antioxidant properties [16]. This is in agreement with the view that most of the antioxidant components share respective functional properties, thus, act additively. Indeed, taking into account the different potencies of the composing tinctures, the antioxidant influence of COMB seems to reflect the sum of the individual antioxidant capacities.

Apart from Carduus marianus, antiproliferative properties were detected in different plants than those with antioxidant effects. The antiproliferative effects of Silybum marianum extracts have been reported earlier [31,32] and there are some notes on such effects from Chelidonium alkaloids [33]. To the best of our knowledge, however, no antiproliferative effect has been previously described for Veratrum and Colocynthis.

From the combination experiments, it seems that the antiproliferative effects are not additive, which is notable, as combinations are usually more effective than the individual tinctures. The most likely explanation for this may be that regulation of cell growth is a complex hierarchical process. Interference with this regulation at different levels will result in non-additive reactions, as seems to be the case for the COMB constituents. For instance, Chelidonium alkaloids such as chelerythrine are known as inhibitors of protein kinase C [34], while flavonolignans from Carduus marianus mainly influence different protein kinases [9, 35, 36] [unpublished observations]. It is interesting, however, that some of the combinations tested, e.g. that of Veratrum and Colocynthis, showed a remarkably high growth inhibition that was not seen to a similar extent with COMB, where all tinctures are combined. This suggests that other tinctures may partly counteract the inhibition. Our results indicate that China might exert such an effect although the tendency was not statistically significant. On the other hand, a slight induction of DNA synthesis by Lycopodium spores has been found in urinary-tract epithelium of the rat [37].

As to biotransformation, only the tincture of Silybum marianum was effective in enhancing the conjugation of DCNB. This is in agreement with results from *in vivo* studies [38]. There is a need for investigations of other biotransformation reactions as it is unlikely that none of the other tinctures should influence this complex function. Nevertheless, the strong enhancement of a phase II reaction as shown for Carduus marianus may contribute to a cancer-preventive potential [9,31] as do the antioxidative and the growth inhibitory functions.

It is well-known that flavonoids and alkaloids, at high concentrations, may have deleterious effects on cells [39,40]. That all three assays for cytotoxicity failed to demonstrate any negative effect of the tested tinctures on the HepG2 cells is important, as it indicates that none of the tinctures contained compounds in concentrations high enough to affect the viability of the cell cultures. Moreover, their combination in COMB does not lead to any enhanced cytotoxicity.

This fact supports the reported safety of the remedy and also points to highly specific effects of the specific components on the cellular functions investigated that may provide various health benefits.

## 5. LITERATURE


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