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ScienceDirect
Phytomedicine 13 (2006) SV 36–44
Phytomedicine
www.elsevier.de/phymed

Radical scavenging and anti-inflammatory properties of STW 5 (Iberogast®) and its components

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Abstract

A combination of ethanolic extracts from nine medicinal plants is successfully used in STW 5 (Iberogast®) for treatment of gastrointestinal disorders. To elucidate possible modes of action, the focus of this study is on antioxidant properties of the phytomedicine STW 5. In fact, functional gastrointestinal diseases, such as non-ulcer dyspepsia (NUD) and irritable bowel syndrome, are often initiated by or correlated to inflammatory processes, where oxidants such as reactive oxygen species (ROS) play a crucial role. Prominent in vivo sources of ROS generation are represented by the enzymes xanthine oxidase (XOD) or myeloperoxidase (MPO). Applying these enzymes in models in vitro, we show that STW 5 and its components possess strong antioxidant activities. Depending on the model investigated, even pro-oxidant activites of single components of STW 5 could be observed. Interestingly, these effects were absent in STW 5, indicating cooperation between the components. Moreover, if one of the component extracts of STW 5 is omitted, the antioxidant activity is reduced. Thus we conclude that all the single extracts combined in STW 5 are of importance for the therapeutic effect, working in concert. The component of STW 5 performing best in vitro differed with the model investigated, respectively, with ROS and ROS generators. In the XOD system, the extracts of lemon balm leaf and peppermint leaf showed the best antioxidant result, whereas concerning MPO driven chlorination reactions, bitter candy tuft extract was the most efficient antioxidant. Best protection against peroxynitrite induced oxidation of methionine like sulfur-compounds exhibited the STW 5 components lemon balm leaf, Matricaria flower and peppermint leaf.

Keywords: Antioxidants; Chlorination; Ethanolic extracts of medicinal plants; Hydrogen peroxide; Hypochlorite; Peroxynitrite; OH-radical; Superoxide radical; Gastorintestinal disorders; STW 5; Iberogast®; *Iberis amara*; Matricaria recutita; Mentha piperita; Carum carvi; Chelidonium majus; Silybum marianum; Angelica archangelica; Glycyrrhiza glabra; Melissa officinalis; Functional dyspepsia (FD); Irritable bowel syndrome (IBS)

Introduction

Gastorintestinal disorders

Inflammatory events often contribute to gastrointestinal diseases, e.g. inflammatory bowel diseases (IBDs) (Madsen et al., 1992; Schumacher et al., 2000; Talley and Spiller, 2002) and also functional gastrointestinal

Abbreviations: ACC, 1-aminocyclopropyl-1-carboxylic acid; STW 5, Iberogast®; IAE, bitter candy tuft extract; IBS, irritable bowel syndrome; KMB, α-keto-γ-methiolbutyric acid; MPO, myeloperoxidase; NUD, non-ulcer dyspepsia; PMN, polymorphonuclear leukocytes; ROS, reactive oxygen species; X, xanthine; XOD, xanthine oxidase

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doi:10.1016/j.phymed.2006.03.017

diseases like non-ulcer dyspepsia (NUD) and irritable bowel syndrome (IBS). Triggering and/or maintaining the disease state (Collins et al., 2001; Gwee et al., 2003; Allescher, 2006), reactive oxygen species (ROS) seem to be involved. Supporting this assumption, reduced antioxidant capacities and an increase in oxidatively damaged DNA have been reported for IBD patients (D'Odorico et al., 2002). ROS include superoxide anion radical, hydrogen peroxide, peroxynitrite, hypochlorous acid/hypochlorite and the extremely reactive hydroxyl radical.

The role of nutrition in treatment of IBDs has been reviewed by Gassull (2004). STW 5 (Iberogast®), a fixed combination of nine herbal extracts is successfully used in the treatment of functional dyspepsia and irritable bowel syndrome (Rösch et al., 2006). Recently, we reported on the potential of scavenging ROS by bitter candy tuft (*Iberis amara* L.) extract (IAE), a component of STW 5. Especially in the myeloperoxidase (MPO) system, IAE exerted striking antioxidant activity (Schempp et al., 2003, 2004). For comparing this activity of IAE to the overall effect of STW 5, all components were investigated for their effect as single extracts in the concentration present in STW 5 (replacing the other eight components by the according volume of 30%(v/v) ethanol). Furthermore, to elucidate the importance of the appropriate component in STW 5, combinations were prepared similar to STW 5, replacing one of the nine components by the appropriate volume of ethanol 30%(v/v). The preparations were investigated in several models, simulating oxidative stress by application of oxidants and targets, which are relevant in vivo. The focus of the study presented here is on the following models: MPO-driven chlorination reactions (Schempp et al., 2004), XOD-mediated generation of superoxide anion radicals (as well as hydrogen peroxide), and peroxynitrite-catalyzed oxidation of methionine residues.

Model systems investigating antioxidant/anti-inflammatory properties

A common feature of our models is to simulate oxidative stress in vitro closely corresponding to in vivo conditions, in particular using oxidants, respectively, ROS, which are relevant in vivo.

MPO-catalyzed chlorination is indicated by ethene formation after chlorination of the detector 1-aminocyclopropane-1-carboxylic acid (ACC). An antioxidant action is indicated by its capacity to inhibit ethene formation by the respective ROS compared to the control reaction (without test compound). This model reaction was either performed with the purified enzyme MPO, or with whole blood samples as a more natural environment. Upon activation with zymosan, polymorphonuclear leukocytes (PMNs) present in the whole blood sample release both, MPO and hydrogen peroxide, into plasma (which contains chloride, 100 mM). MPO catalyses the formation of the strong microbicidal oxidant hypochlorous acid ($pK_a = 7.5$) in the presence of hydrogen peroxide and chloride, according to the following equation:

$$\begin{aligned} &H_2O_2 + Cl^- + H^+ \xrightarrow[pH=6.0]{MPO} HOCl + H_2O, \\ &ACC + HOCl \rightarrow \text{ACC-chloramine} + H_2O \\ &\qquad \rightarrow \text{ethene} + \text{products}. \end{aligned} \quad (1)$$

Under the conditions in our model (pH 6.0), MPO converts given hydrogen peroxide (25 μM) entirely into $HOCl/OCl^-$. To prove scavenging of HOCl by STW 5 and its components, sodium hypochlorite (25 μM) was added directly instead of $MPO/Cl^-/H_2O_2$. In this model, inhibition of ethene production from ACC by different plant extracts is reflecting either reactivity with HOCl, i.e. competition with ACC for this oxidant, or direct influence on the enzyme, MPO. With these systems, chlorination by purified MPO as well as by activated leukocytes in whole blood samples, inflammatory oxidative stress was simulated and screened for its attenuation by the mentioned components of STW 5.

XOD generates superoxide anion radicals during oxidation of xanthine into uric acid. Concerning inflammation, superoxide anion radicals are also produced by activated leukocytes during the oxidative burst. In the XOD model, superoxide anion radicals are detected by the conversion of hydroxylamine into nitrite:

$$\begin{aligned} &\text{Xanthine} + O_2 + H_2O \xrightarrow[pH=7.4]{XOD} \text{uric acid} + O_2^{\bullet -}/H_2O_2 \\ &O_2^{\bullet -} + NH_2OH \rightarrow {}^-OONHOH \rightarrow NO_2^- + H_2O. \end{aligned} \quad (2)$$

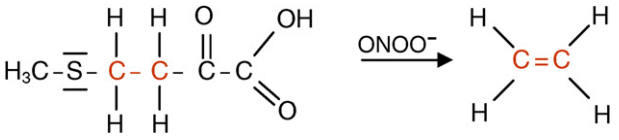
After incubation, accumulation of nitrite is quantified by azo dye formation in acidic environment (sulfanilic acid/α-naphtyl ethylenediamine), well known as the Gries reaction. Ethanol, a good OH-radical scavenger, does not interfere with nitrite formation, indicating that OH radicals are not essential for oxidation of hydroxylamine into nitrite. Superoxide dismutase (50 units/ml) completely inhibits nitrite formation from hydroxylamine catalyzed by X/XOD.

Peroxynitrite is a strong oxidant and nitrating agent, oxidizing preferentially reduced sulfur compounds like thiols or methionine residues. During inflammation, it is formed by the rapid reaction of superoxide anion radical with nitrogen monoxide (produced by NO synthases from arginine). We used ethene formation from α-keto-γ-methiolbutyric acid (KMB) as a sensitive test for peroxynitrite mediated damage of methionine

like residues:

$$O_2^{\bullet -} + NO^{\bullet} \xrightarrow{K=6.2\times10^{9}\,M^{-1}\,s^{-1}} {}^{-}OONO,$$

$$^{-}OONO + KMB \rightarrow \text{ethene} + \text{products}.(3)$$



While OH radical-induced formation of ethene from KMB is completely blocked by ethanol (>100 mM), peroxynitrite-mediated ethene formation is still observable. To get familiar with the concentration values, it is mentionable that 170 mM ethanol is equivalent with a ethanol solution of 1% (v/v).

The "STW 5-bioactivity" measured in our models is the result of the combined pro- and antioxidant activities of the single-extract ingredients, working in concert. The advantage of this strategy, instead of HPLC determination of ingredients, is to predict their activity in the extract environment when applied as a drug in situations of pathological oxidative stress.

Materials and methods

STW 5 and referring plant extracts

The fresh plant extract of bitter candy tuft and drug extracts of milk thistle fruit, caraway fruit, chamomile flower, liquorice root, lemon balm leaf, peppermint leaf, angelica root, greater celadine herb and the "reference" STW 5 have been obtained from Steigerwald Arzneimittelwerk GmbH, Darmstadt, Germany. In STW 5, these nine extracts are combined. Two preparations were provided by Steigerwald: the single extract in the same concentration as in STW 5, but filled up with 30% (v/v) ethanol. An incomplete STW 5 preparation, where the mentioned part is omitted and replaced by the same volume of 30% (v/v) ethanol. Furthermore, Steigerwald delivered lyophilisates of the single extracts, where ethanol 30% (v/v) was used as solvent.

Chemicals

ACC, KMB, NH_2OH, $NaNO_2$, sulfanilamide, α-naphtylethylenediamine, sodium hypochlorite (≥5% active chlorine), superoxide dismutase, xanthine and uric acid, zymosan were purchased from Sigma-Aldrich Chemie (Munich, Germany). EDTA, H_2O_2, HCl 32%, buffer salts and saccharose were obtained from Merck (Darmstadt, Germany). MPO from human leukocytes was purchased from Calbiochem (Merck Biosciences, Nottingham, UK). Xanthine oxidase (XOD) and catalase were from Roche Diagnostics (Mannheim, Germany). Peroxynitrite was produced according to Beckman et al. (1990). After dilution in 0.1 M NaOH, its concentration was measured photometrically using $\varepsilon_{302nm} = 1670\,M^{-1}\,cm^{-1}$. The concentration of stock solutions of H_2O_2 ($\varepsilon_{240nm} = 39.6\,M^{-1}\,cm^{-1}$) and of NaOCl ($\varepsilon_{292nm} = 350\,M^{-1}\,cm^{-1}$ at pH 12) were also determined photometrically.

Instruments

Gas chromatograph: Varian 3400cx with flame ionisation detector and Shimadzu R6a Integrator. The GC was set to the following temperatures: injector and column: 80 °C; FID:225 °C. Separation of the headspace samples were carried out by direct injection on a varian aluminum oxide column (0.125 in × 60 cm). The carrier gas was N_2 (type 5.0; degree of purity 99.999%, flow rate 25 ml/min) and the FID-gases were H_2 (type 5.0; degree of purity 99.999%, flow rate 25 ml/min) and synthetic air (flow rate 250 ml/min). A diluted standard of ethene gas (mixture of 7.27 mg ethene/m^3 synthetic air: 1 ml = 259 pmol at 1 bar) is used for calibration of the FID. Gases were purchased from Messer Griesheim, Darmstadt. Spectrophotometer: Kontron Instruments Uvikon 922.

Test systems and methods

The xanthine/xanthine-oxidase system

XOD generates $O_2^{\bullet -}$, H_2O_2 and uric acid using xanthine and molecular oxygen as substrates. XOD activity is characteristic for reperfusion injury after ischemic events. It is detectable as nitrite production from NH_2OH catalyzed by superoxide radicals, which can be decreased by superoxide scavengers or XOD inhibitors (Schempp et al., 2003). An assay contains in a final volume of 2 ml: 0.1 M phosphate buffer pH 7.4, NH_2OH 1 mM, 0.5 mM xanthine, XOD 0.04 U/ml, STW 5 samples (100 µl for K1–K9; 5 µl for STW 5 and Pl (1:10 diluted). After incubation for 30 min at 37 °C, nitrite analysis (Gries reaction) was performed with 300 µl aliquots of the samples, which were placed into 1.5 ml cuvettes filled with 300 µl of sulfanilamide (1% (w/v) in 8 N% HCl). Azo dye formation was started by addition of 300 µl α-napthylethylenediamine (0.2% (w/v), and after further 15 min (for complete color formation) absorbance ($A_{540\,nm}$) was measured. A decrease in $A_{540\,nm}$ compared to the basic reaction is due to less nitrite formed, indicating superoxide scavenigng activity of the tested compound, provided that inhibition of XOD can be ruled out and nitrite-dependent azo dye formation is not interfered.

Activity of XOD was quantified by HPLC analysis of xanthine and uric acid (Waters HPLC consisting of

600-pump, 717-autosampler and 996-PDA detector, column Merck Cartridge 125 × 4 mm, Lichrospher® 60 RP-select B, column temperature 35 °C, eluent NaH_2PO_4 50 mM and methanol (grad. grade) for the washing step of the column after each injection; flow rate 1 ml/min, sample volume 20 µl. Retention times: xanthine 3.0 min, uric acid 4.0 min.) For XOD-activity determination, the XOD reaction was performed in the presence or absence of the investigated plant extract. The enzyme reaction was stopped after 10 min by addition of 50 µl 10 M HCl. Influence of the extracts on XOD activity was expressed by comparing amounts of uric acid produced as well as xanthine consumed. An assay contains in a final volume of 1 ml: 0.1 M phosphate buffer pH 7.4, 0.5 mM xanthine, XOD 0.04 U, amount of STW 5 samples (100 µl for K1–K9; 5 µl for STW 5 and Pl). After exactly 10 min of incubation at 37 °C, HCl was added as already described.

Peroxynitrite, $ONOO^-$

$ONOOH/ONOO^-$ is detected by ethene formation during oxidation of KMB. A decrease of ethene production in the presence of the investigated substance is equivalent to detoxification of peroxynitrite (Schempp et al., 2003; Schempp et al., 2000). Interestingly, peroxynitrite-mediated oxidation of KMB into ethene is marginally decreased by ethanol (a potent OH-radical scavenger, 1% EtOH = 170 mM), whereas OH radical induced ethene formation is completely blocked. Thus, peroxynitrite is only negligible forming OH radical via homolytic decomposition into NO_2 radical and OH radical. Peroxynitrite was synthesized according to Beckman et al., 1990. The alkaline peroxynitrite solution was checked by $\varepsilon_{302\,nm} = 1670\,M^{-1}\,cm^{-1}$ after dilution with 0.1 M NaOH. An assay contains in 2 ml final volume: 0.1 M phosphate buffer pH 7.4, 1 mM KMB, 50 µM ONOOH (20 µl of 5 mM $ONOO^-$ in 0.1 M NaOH) and STW 5 samples (300 µl K1-K9). After incubation of 30 min at 37 °C, 1 ml aliquots of the headspace of the samples were withdrawn with gas-tight syrings and analyzed by gas chromatography for their content of ethene.

The hypochlorite/ACC system – MPO catalyzed formation of HOCl

Purified MPO

In vivo, MPO is responsible for HOCl formation. In our model system purified MPO from human leukocytes is used under optimal conditions concerning HOCl formation. In the presence of 100 mM NaCl and 25 µM hydrogen peroxide in a volume of 2 ml buffered at pH 6 × 50 mM phosphate, 0.25 µg/ml MPO converts entirely all hydrogen peroxide into HOCl within 45 min at 37 °C. Produced HOCl is detected by the oxidation of ACC into ethene, which is specific for HOCl or HOBr (Schempp et al., 2003; von Kruedener et al., 1995). Whether an antioxidant activity is due to MPO inhibition or detoxifcation of HOCl is checked by testing the extracts to protect ACC from oxidation by sodium hypochlorite. The test system contains in a final volume of 2 ml: 50 mM phosphate buffer pH 6.0, 1 mM ACC, 25 µM HOCI, STW 5 samples (K1–K9, 300 µl). After incubation of 45 min at 37 °C in gas-tight-sealed reaction tubes, 1 ml gas of the headspace has been retained with a gas-tight syringe and analyzed gas chromatographically for its ethene content.

Activated leukocytes in whole blood

EDTA blood (1 mg/ml blood) was obtained from healthy donors (male and female, age 20–30 years). Addition of zymosan to whole blood samples activates neutrophilic granulocytes, which release the enzyme MPO into the extracellular environment during the degranulation process. In turn, the oxidative burst product hydrogen peroxide of the zymosan-activated neutrophils catalyses MPO-dependent HOCl formation, which is indicated by ethene release from ACC. The assay contains in a total volume of 2.00 ml: 1 ml blood (freshly withdrawn from healthy donors), 5 mg zymosan, 1 mM ACC, and the indicated amounts of extracts. All substances were solubilized in Sorenson PBS-buffer pH 7.4 (containing plasma amounts of Ca^{2+} and Mg^{2+}). Amount of extract added: 50 µl in 30% (v/v) ethanolic solution containing the indicated amounts of dry weight present in the original STW 5. After incubation for 45 min at 37 °C in gas-tight sealed test tubes in a water bath in the dark, 1 ml gas of the headspace was retained with a gas-tight syringe and analyzed gas chromatographically for its amount of ethene. Viabilities of neutrophils were assessed by trypan blue dye exclusion. Hemolysis of the whole blood samples was tested by centrifugation (1200 *g* for 10 min) and UV–vis photometry of the reaction mixtures after incubation. In case of hemolysis, the supernatant (blood plasma) should be colored red due to free hemoglobin. Hemoglobin absorbance of the supernatant was measured spectrophotometrically (Soret absorbance at 403 nm): None of the tested extracts showed any cytotoxic or hemolytic potential. As a positive control, hypotonic conditions were induced by adding 0.5 ml bidest instead of PBS to 1 ml whole blood to a final volume of 2 ml, resulting in an obviously red color of the supernatant after centrifugation of the sample.

Statistics

The results shown are means of at least three individual experiments ($n = 3$), the standard deviations are given as σ_{n-1}.

Results

Influence of STW 5 and its components on superoxide catalyzed (X/XOD) oxidation of NH_2OH into nitrite

Superoxide is a ROS relevant in inflammatory reactions. In the model used here, superoxide is generated by X/XOD and the influence of STW 5 and its single components is investigated. The STW 5 components are ethanolic extracts (30% ethanol) of:

K1 *Iberis amara*	K4 *Matricaria recutita*	K7 *Mentha piperita*
K2 *Silybum marianum*	K5 *Glycyrrhiza glabra*	K8 *Angelica archangelica*
K3 *Carum carvi*	K6 *Melissa officinalis*	K9 *Chelidonium majus*

In the "K" preparations, eight from the nine components of STW 5 are omitted and replaced by the according volume of ethanol 30% (v/v). As shown in Fig. 1, all components are active in decreasing amounts of detected superoxide. The activity of the components can be rated in three categories:

high antioxidant activity: K6, K7,
medium antioxidant activity: K2, K3, K4, K8, K9,
low antioxidant activity: K1, K5.

A special focus of our study was on activity of IAE (K1), an important component of STW 5. Therefore a "placebo" of STW 5 was prepared by replacing the IAE part of STW 5 by the according volume of 30% ethanol (v/v). It is obvious that compared to the K preparations, higher dilutions are necessary to get into the valid range of our test system. In a final concentration of 1:2000, placebo was only marginally less active than STW 5, indicating a minor role of IAE concerning antioxidant activity of STW 5 against superoxide scavenging (Fig. 1, on the right site). None of the extracts decreased XOD activity significantly as measured by HPLC analysis of amounts of uric acid formed and amounts of xanthine consumed. Thus, the activity measured in the hydroxylamine test is due to a decrease of superoxide and thus radical scavenging activity of the STW 5 components.

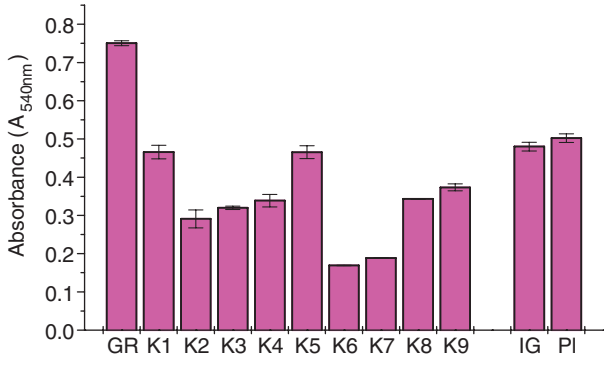


Fig. 1. Effect of single components (K1–K9) of STW 5 on X/XOD-catalyzed superoxide fromation in vitro. Superoxide is detected by oxidation of hydroxylamine into nitrite, quantified after azo dye formation (Gries reaction). In "K" the according single component is concentrated as in STW 5, but the other eight extracts present in STW 5 are omitted and replaced by the according volume of ethanol 30% (v/v). Pl (placebo) is a preparation analog to STW 5, with the exception that from the nine components of STW 5, *Iberis amara* extract (IAE) is omitted and replaced by the appropriate volume of ethanol 30% (v/v). (GR = basic reaction, IG = STW 5; Pl = (STW 5 without IAE)).

Influence of STW 5 and its components on peroxynitrite-mediated ethene formation from KMB

Peroxynitrite is a strong oxidizing and nitrating agent. Here we applied synthesized peroxynitrite (synthesis of peroxynitrite according to Beckman et al., 1990) and the methionine derivative KMB as target. Ethene formation from KMB indicates oxidation by peroxynitrite. As illustrated in Fig. 2, all components of STW 5 were able to decrease peroxynitrite-mediated ethene production from KMB. In this model, the activity of the components could be rated:

high antioxidant activity: K6, K4, K7,
medium antioxidant activity: K8, K9, K3, K2, K5,
low antioxidant activity: K1.

Interestingly, K1 (IAE) again showed only low activity compared to the other components of STW 5. However in the model described next, K1 is the most active. Thus, antioxidant activity is strictly dependent on the selected model used, and selection of the model(s) to characterize antioxidant activity is worth emphasizing.

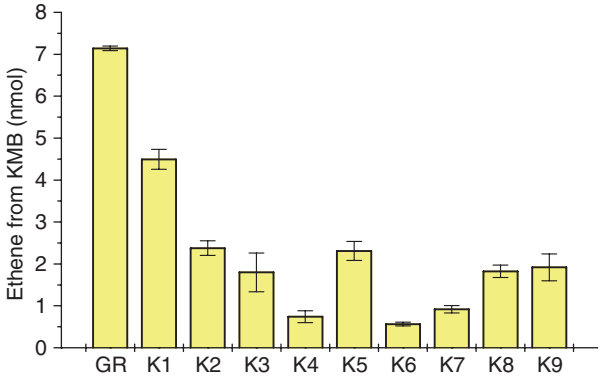


Fig. 2. Influence of single components (K1–K9) of STW 5 on peroxynitrite catalyzed ethene release from KMB. In "K" the according single component is concentrated as in STW 5, but the other eight extracts present in STW 5 are omitted and replaced by the according volume of ethanol 30% (v/v).

Inhibition of the MPO-catalyzed chlorination of ACC by STW 5 and its components

The model of MPO-catalyzed chlorination is simulating inflammatory events due to activated leukocytes, especially polymorphnuclear neutrophil (PMNs) granulocytes). HOCl formed by MPO catalysis is detected by the non-proteinogenic amino acid ACC. HOCl reacts with ACC to instable ACC chloramine, which decomposes under formation of ethene. A clear loss of antioxidant capacity in the MPO system is detectable compared to the whole drug STW 5 (Fig. 3), when IAE is omitted from the preparation of STW 5 (STW 5–IAE = placebo). Furthermore, the contribution of IAE to performance of STW 5 in this system is emphasized by comparsion of the according dose response curves, as shown by the different IC_{50} values. Comparing the single components (K1–K9), it turned out that K1 and K4 were the most active, as shown in Fig. 4a. Moreover, even pro-oxidant acitvities were detectable for K3, K7 and K9. As already published (Schempp et al., 2004), these proxidant effects could clearly be attributed to the hydrogen peroxide content of these preparations (K3, K7, K9), resulting in additional formation of HOCl and thus higher amounts of ethene from ACC. Under the conditions used in our model, HOCl formation is linearly dependent on hydrogen peroxide and converted completely into HOCl in the range from 1 to 50 µM H_2O_2. Hydrogen peroxide may represent an autoxidation product of the respective extracts (K3, K7, K9). Preincubation with catalase completely abolished pro-oxidant activities of these extracts. Alltogether, three categories can be differentiated comparing activties of K1–K9 (Fig. 4a):

antioxidant activity: K1, K4, K2, K6, K5,
no activity: K8,
pro-oxidant activity: K3, K7, K9.

It should be noted that dry weight content of K4 (13.8 mg/ml) is much higher than of K1 (1.95 mg/ml), so that related to their dry weight, K1 is much more active. The extracts from caraway fruit (K3) or peppermint leaf (K7) exhibit their stimulatory effects, as already shown (Fig. 4a). In Fig. 4b is illustrated that addition of both extracts did not further increase the reactivity, indicating peroxide saturation of the system. Addition of the inhibitory extracts K1 or K4, either single or in combination, down regulated these stimulatory effects of K3, K7 or both, added again either single or in combination (Fig. 4b). However, their activities were far away from the potent antioxidant effect of the whole drug STW 5 (Fig. 4b, red box), indicating the importance of all components in exerting the antioxidant effect in the MPO-chlorination system, working in concert.

Zymosan induces activation (respiratory burst/degranulation/phagocytosis) of neutrophilic granulocytes in whole blood, releasing the enzyme MPO into the extracellular environment. Driven by H_2O_2 from the oxidative burst, MPO forms HOCl in the presence of chloride (100 mM chloride is present in whole blood). Thereby, HOCl/hypochlorite production is indicated by the formation of ethene in the presence of the online detector ACC. As shown in Fig. 4c, the excellent performance of K1 could be confirmed in this more complex system (compared to purified MPO). There are three categories of concentration-dependent effects of the investigated extracts in the whole blood model, concerning their inhibition of ethene release from ACC:

high antioxidant activity: K1,
medium antioxidant acitivity: K4, K6, K7,
low antioxidant activity: K2, K3, K5, K8, K9.

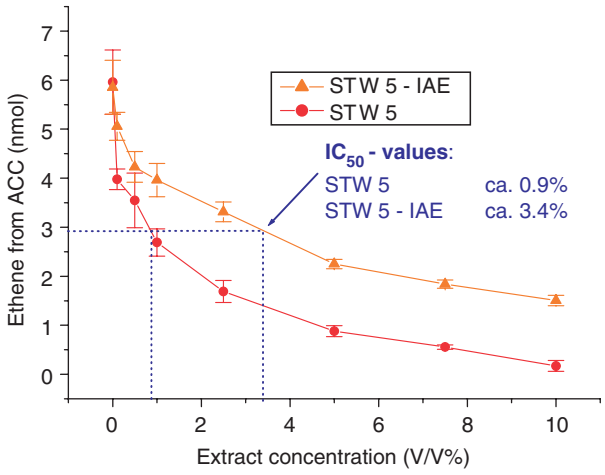


Fig. 3. Effect of replacing the IAE part of STW 5 by the according volume of ethanol 30% (v/v) on inhibition of MPO catalyzed ACC formation by HOCl.

Discussion

In this study, corresponding effects of nine ethanolic herbal extracts composing the drug STW 5 are reported. Focussing on relevant oxidants in vivo, three in vitro model reactions were chosen to characterize antioxidant performance of STW 5 and its components.

(1) radical scavenging activity concerning superoxide generated by X/XOD,
(2) scavenging of peroxynitrite determined by ethene formation from KMB,
(3) HOCl scavenging activity or MPO inhibition in the ACC-ethene model with purified MPO or whole blood.

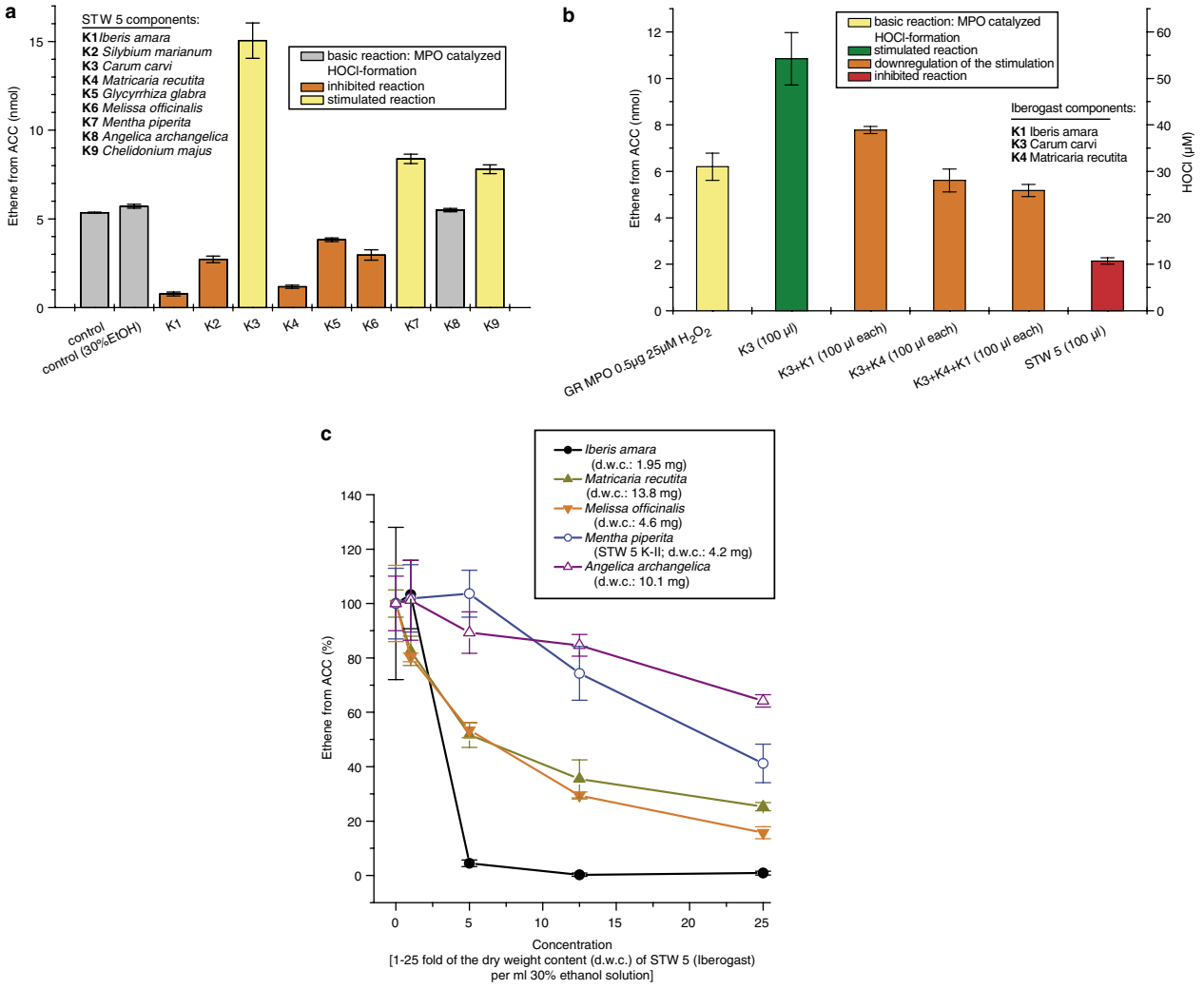


Fig. 4. (a) Influence of single extract components of IG on MPO catalyzed HOCl formation, measured as ethene release from ACC. (b) Down regulation of the stimulating effect of the extract of caraway fruit by the single components bitter candy tuft (K1) and chamomile flower (K4), in comparison to the effect of the complete drug IG. (c) Influence of IG components on ethene formation from ACC, driven by HOCl produced by zymosan activated PMNs in whole blood. In final 2.0 ml: ACC 1 mM, zymosan 5 mg, EDTA whole human blood (1 mg EDTA/ml blood), Soerenson buffer, amounts of extracts as indicated.

All tested oxidants (superoxide, peroxynitrite, HOCl) are relevant in vivo during inflammatory reactions. Thus, the measured activities of STW 5 and its components most probably represent modes of action in vivo. Anti-inflammatory activities of STW 5 already have been reported in other models (Okpanyi, 1993; Khayyal et al., 2006; Michael et al., 2006), but not with this special focus on oxidants and targets. In the MPO model the capacity is tested to protect against tissue damage mediated by MPO generated HOCl. Antioxidant activity can be exerted in two ways: On the one hand HOCl can be scavenged, on the other hand the generation of HOCl can be decreased or blocked by inhibition of the catalytic activity of MPO. Online detection of low steady-state concentrations of HOCl (in the µM range) is one advantage of using ethene formation from ACC. From the results it is concluded that IAE extract of STW 5 plays a key role concerning downregulation of MPO-driven inflammatory reactions such as chlorination or HOCl formation. This is exclusively demonstrated replacing the IAE content of STW 5 (Fig. 3) by the same volume ethanol (30% v/v) and by testing IAE alone (Schempp et al., 2003) in the purified MPO as well as in the whole blood model (Schempp et al., 2004, Fig. 4a and c).

In contrast, IAE showed the lowest activity, compared to the other eight herbal components of STW 5, in models testing scavenging activities towards superoxide or peroxynitrite. These facts demonstrate the importance of all herbal extract components of STW 5, performing as a good antioxidant in all applied model reactions, simulating inflammatory reactions.

Furthermore, the importance to use more than one test system to characterize antioxidant activity is shown by the different results, when different models concerning oxidants and targets are used. Moreover, the test systems used should simulate relevant oxidants, also occurring in vivo. Concerning significance, the use of relatively stable and colored radicals to characterize antioxidant activity is questionable, although their measurement is easy. DPPH radical as well as ABTS radical represent such stable radicals, which are widely used to determine antioxidant capacities of compounds in vitro. However, in vivo such stable radicals are an exception. DPPH or ABTS radical behave more like antioxidants (the antioxidant is for both the reduced form of the radical, converted by an oxidant into the stable radical with restricted and low activity) which can be regenerated into the antioxidant form by reducing agents like phenols. Besides the reductant potential, the efficiency to reduce such radicals is determined by the affinity (stereochemical parameters, charge, structure) and thus by kinetics of the interaction with the radical, as well as the fate of reaction products and their reactivity. In addition, the most important parameter is that these radicals do not add molecular oxygen promoting radical chain reactions, as it is the case in lipid peroxidation.

One important radical in vivo is the superoxide anion radical. In contrast to the radicals described above, superoxide is only a weak oxidizing agent. In the slightly acidic microenvironment in the proximity of membranes it can be protonated ($HOO^{\bullet}$), thereby changing its oxidant potential inducing lipid peroxidation. The more crucial problem of superoxide is represented by its reducing property and high affinity towards Fe^{3+} and towards nitrogen monoxide. In the first case, Fe^{2+} is formed which can promote the so-called Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$) producing the strong oxidant OH radical. To keep these activities on a low level, Fe^{3+} is efficiently bound and stored in metalloproteins (Transferrin and Ferritin). Free Fe^{2+} is immediately oxidized to Fe^{3+} by the ferroxidase caeruloplasmin. In the second case peroxynitrite is formed, a strong oxidizing and nitrating compound. To down regulate the extent of such reactions superoxide dismutase is most important (but not sufficient) and thus essential for all aerobes.

The STW 5 components lemon balm leaf and peppermint leaf exerted the highest superoxide scavenging activity in the X/XOD–hydroxylamine test. Both components of STW 5 showed good protection of KMB from oxidation by peroxynitrite. In addition lemon balm leaf extract was also quite active in the MPO-dependent systems, where bitter candy tuft showed the best protection against MPO-catalyced HOCl formation.

In conclusion, STW 5 showed remarkable oxidant scavenging and antiinflammatory properties in the models used here. A similar effect in vivo has to be assumed. Furthermore, it was shown that not only one marker substance or component is responsible for the overall performance, but that the cooperation of all compounds is accounting for the efficacy of STW 5, supporting the multi-target therapy concept.

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