Extraction of *Waltheria indica* compounds via accelerated solvent extraction and identification of relevant molecule classes for optimized cyclooxygenase-2 inhibition

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Michael Termer

aus **Dzezkasgan / Kasachstan**

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Erstgutachter:	Prof. Dr. Cornelia M. Keck
Zweitgutachter:	Prof. Dr. Jürgen Hemberger
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Extraction of *Waltheria indica* compounds via accelerated solvent extraction and identification of relevant molecule classes for optimized cyclooxoginase-2 inhibition

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Michael Termer

from **Dzezkasgan / Kasachstan**

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First Supervisor:	Prof. Dr. Cornelia M. Keck
Second Supervisor:	Prof. Dr. Jürgen Hemberger
Date of Submission:	01.12.2021
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ERKLÄRUNG

Ich versichere, dass ich meine Dissertation

„EXTRACTION OF WALTHERIA INDICA COMPOUNDS VIA ACCELERATED SOLVENT EXTRACTION AND IDENTIFICATION OF RELEVANT MOLECULE CLASSES FOR OPTIMIZED CYCLOOXOGINASE-2 INHIBITION“

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I declare that this thesis titled

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was prepared by myself independently without unauthorized assistance and have not used any sources other than those expressly designated by me. All quotations taken over in full or in part are marked as such.

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1. Abstract

Inflammation is the bodies response to infection or tissue injury in order to restore and maintain homeostasis. Prostaglandin E2 (PGE-2) derived from arachidonic acid (AA), via up-regulation of cyclooxygenase-2 (COX-2) is a key mediator of inflammation and can also be induced by several other factors including stress, chromosomal aberration, or environmental factors. Targeting prostaglandin production by inhibiting COX-2 is hence relevant for the successful resolution of inflammation.

Waltheria indica is a traditional medicinal plant with reported anti-inflammatory properties whose extracts have demonstrated COX-2 inhibitory activity. However, the compounds responsible for the activity were merely assumed and, in most cases, unknown or assigned to molecules, such as tiliroside, whose content in the tested extracts during the investigations was unknown. For the preparation of extracts with effective anti-inflammatory properties, characterization of these substances is vital.

In this thesis, this challenge was addresses by characterizing of substances responsible for the COX-2 inhibitory activity in the extracts and the generation of a prediction models to quantify the COX-2 inhibitory activity without the biological testing. The inhibitory potential of the probes against the COX-2 enzyme was assessed with a fluorometric COX-2 inhibition assay. The results obtained demonstrated that *Waltheria indica* extracts inhibited the inflammatory key mediator COX-2 with the activity related to the extraction parameters governing the composition of the extract. The examinations on tiliroside activity and content in the extracts revealed that although tiliroside demonstrated COX-2 inhibitory activity in a concentration-dependent manner, the content of tiliroside in the extracts was not sufficient to contribute to the observed activity with the extracts. For the identification of contributors to the observed COX-2 inhibitory activity, an extract was separated into fractions by means of centrifugal partition chromatography (CPC) and their COX-inhibitory activity evaluated. The characterization of compounds in fractions with highest COX-2 inhibitory activity were done by high resolution mass spectrometry (HPLC-MS/MS). It was found that these fractions contained alpha-linolenic acid, linoleic acid and oleic acid, identified and reported for the first time in *Waltheria indica* leaf extracts. After analyzing their content in extracts obtained varying extraction parameters, it could be demonstrated that these fatty acids contribute up to 41% to COX-2 inhibition observed with *Waltheria indica* extract. Additional quantification of phytochemicals in the extract fractions established that substances from the group of steroidal-saponins and triterpenoid-saponins also contribute to the COX-2 inhibitory activity.

Based on the content of compounds contributing to COX-2 inhibition two mathematical models were successfully developed both with a root mean square error (RMSE) = 1.6% COX-2 inhibitory activity, demonstrating a high correspondence between predicted versus observed values. The results of the predictive models further suggest that the compounds contribute to COX-2 inhibition in the order linoleic acid > alpha linolenic acid > steroidal-saponins > triterpenoid-saponins. Based on the developed mathematical models in this project, a more targeted development of extraction procedures is possible in order to obtain *Waltheria indica* extracts with improved anti-inflammatory properties related to the inhibition of COX-2. Furthermore, the transfer of the approach presented in this work to the prediction of other biological endpoints would be of great interest for future studies.

2. Zusammenfassung

Entzündungen sind die Reaktion des Körpers auf Infektionen oder Gewebeverletzungen, um die Homöostase wiederherzustellen und zu erhalten. Prostaglandin E2 (PGE-2), das aus Arachidonsäure (AA) über eine Hochregulierung der Cyclooxygenase-2 (COX-2) gewonnen wird, ist ein wichtiger Mediator von Entzündungen und kann auch durch verschiedene Faktoren wie Stress oder Umweltfaktoren ausgelöst werden. Die gezielte Steuerung der Prostaglandinproduktion durch die Hemmung von COX-2 ist daher für die erfolgreiche Behebung von Entzündungen von großer Bedeutung.

Waltheria indica ist eine traditionelle Heilpflanze mit nachgewiesenen entzündungshemmenden Eigenschaften, deren Extrakte eine hemmende Wirkung auf COX-2 gezeigt haben. Die für die Aktivität verantwortlichen Verbindungen wurden jedoch lediglich vermutet und waren in den meisten Fällen unbekannt oder wurden Molekülen wie Tilirosid zugeordnet, deren Gehalt in den getesteten Extrakten während der Untersuchungen unbekannt war. Für die Herstellung von Extrakten mit wirksamen entzündungshemmenden Eigenschaften ist die Charakterisierung dieser Substanzen unerlässlich.

In dieser Arbeit wurde diese Herausforderung durch die Charakterisierung der für die COX-2 hemmende Aktivität verantwortlichen Substanzen in den Extrakten und die Erstellung eines Vorhersagemodells zur Quantifizierung der COX-2 hemmenden Aktivität ohne biologische Tests angegangen. Das hemmende Potenzial der Proben gegen das COX-2-Enzym wurde mit einem fluorometrischen COX-2-Hemmtest bewertet. Die erzielten Ergebnisse zeigten, dass die Extrakte von *Waltheria indica* den Entzündungsmediator COX-2 hemmen, wobei die Aktivität von den Extraktionsparametern abhängt, die ebenfalls die Zusammensetzung des Extraktes bestimmen. Die Untersuchungen der Tilirosid-Aktivität und des Tilirosid-Gehalts in den Extrakten ergaben, dass Tilirosid zwar eine konzentrationsabhängige COX-2-hemmende Aktivität zeigte, der Gehalt in den Extrakten jedoch nicht ausreichte, um zur beobachteten Aktivität der Extrakte beizutragen. Zur Identifizierung der Substanzen, die zur COX-2-hemmenden Wirkung beitragen, wurde ein Extrakt mittels Zentrifugalpartitionschromatographie (CPC) in Fraktionen aufgetrennt und deren COX-hemmende Wirkung analysiert. Die Charakterisierung der Verbindungen in den Fraktionen mit der höchsten COX-2-hemmenden Aktivität erfolgte durch hochauflösende Massenspektrometrie (HPLC-MS/MS). Es wurde festgestellt, dass diese Fraktionen alpha-Linolensäure, Linolsäure und Ölsäure enthalten, die zum ersten Mal in *Waltheria indica* Blattextrakten identifiziert und beschrieben wurden. Nach der Analyse ihres Gehalts in den Extrakten konnte nachgewiesen werden, dass diese Fettsäuren bis zu 41 % zur COX-2-Hemmung beitragen, die mit *Waltheria indica* Extrakten beobachtet wurde. Eine zusätzliche Quantifizierung der Phytochemikalien in den Extraktfraktionen ergab, dass Substanzen aus der Gruppe der Steroid-Saponine und Triterpenoid-Saponine ebenfalls zur COX-2-hemmenden Aktivität beitragen.

Auf der Grundlage des Gehalts an Verbindungen, die zur COX-2-Hemmung beitragen, wurden erfolgreich zwei mathematische Modelle entwickelt, die beide einen mittleren quadratischen Gesamtfehler von 1,6 % für die COX-2-hemmende Aktivität aufwiesen und damit eine hohe Übereinstimmung zwischen vorhergesagten und beobachteten Werten. Die Ergebnisse der Vorhersagemodelle deuten ferner darauf hin, dass die Verbindungen zur COX-2-Hemmung in der Reihenfolge Linolsäure > alpha-Linolensäure > Steroid-Saponine > Triterpenoid-Saponine beitragen.

Die entwickelten mathematischen Modelle ermöglichen eine gezieltere Entwicklung von Extraktionsverfahren, um Extrakte aus *Waltheria indica* mit verbesserten entzündungshemmenden Eigenschaften im Zusammenhang mit der Hemmung von COX-2 zu erhalten. Darüber hinaus wäre die Übertragung des in dieser Arbeit vorgestellten Ansatzes auf die Vorhersage anderer biologischer Endpunkte für zukünftige Studien von großem Interesse.

3. Introduction

3.1. Inflammation

Inflammatory disorders, including various types of rheumatic conditions, are a major contributor to human morbidity. Inflammation is a defensive response of the body as a cyclic, self-stimulating process designed to combat infection or tissue injury [1,2]. The inflammatory process is accompanied by the release of pro-inflammatory cytokines, prostaglandins, and the formation of reactive oxygen species (ROS) [3]. Crosstalk between different cellular components regulates local immune responses to maintain and restore homeostasis [4,5]. Dysregulated inflammatory responses contribute to chronic disease progression and inflammatory chronic or auto-immune disorders [6,7].

Important modulators of inflammation are nuclear factor kappa B (NF-kB), lipoxygenase (LOX) and cyclooxygenase (COX), with NF-kB activating the expression of LOX or COX [8,9]. Both enzymes are part of the arachidonic acid (AA) metabolism a main cellular process for mediating inflammation exhibiting a catalytic effect. The COX-1 and COX-2 enzymes are isozymes, and in normal human skin COX-1 is present through the epidermis whereas COX-2 localizes mainly in suprabasal keratinocytes [10-12]. While COX-1 is involved in homoeostatic processes and expressed constitutively in most tissues, the pro-inflammatory COX-2 is an inducible isoform and mainly produced in inflamed tissues and together with COX-1 responsible for the conversion of AA to prostaglandin G2 (PGG_2) and further to prostaglandin H2 (PGH_2). PGH_2 is converted into prostanoids thromboxane A2 (TXA_2), prostaglandin E2 (PGE_2), prostacyclin (PGI_2), prostaglandin D2 (PGD_2), and prostaglandin F2 alpha ($PGF_{2\alpha}$) by individual prostaglandin synthase enzymes [13-16]. Schematic representation of the prostaglandin synthesis is illustrated in Figure 1.

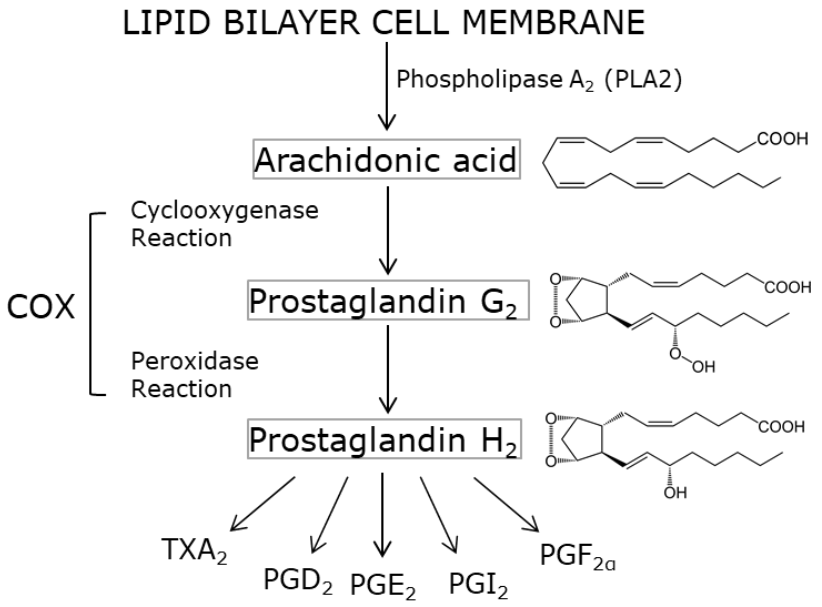

Figure 1: Schematic representation of the prostaglandin synthesis. Arachidonic acid, a C20 polyunsaturated fatty acid, is released from membrane phospholipids by PLA2, which is activated by diverse stimuli. Arachidonic acid is converted to the endoperoxide PGG2 and further reduced to PGH2 by the action of COX enzymes. PGE2 is formed from PGH2 by PGE synthases. Additionally the prostanoids TXA2, PGD2, PGI2 and PGF2α are formed by their specific isomerases.

To maintain and restore homeostasis in human skin, different cellular components regulate local immune responses thorough crosstalk [2,9]. The initiation and the maintenance of the inflammation is carried out by pro-inflammatory mediators. Once the instigating factor is removed activity is balanced out by the anti-inflammatory mediators responsible for limiting the inflammatory response [10,11]. Several factors including stress, chromosomal aberration, or environmental factors lead to excessive production of PGE2 derived from arachidonic acid, via up-regulation of COX-2, consequently leading to inflammatory mediated diseases [12-16]. Increased PGE2 levels lead to increased production of proinflammatory cytokines like interleukin-6 (IL-6) with inflammatory disease activities directly linked to the IL-6 concentration [17-19]. Interaction of COX-2 inhibitors on the level of PGE2 and IL-6 are shown in Figure 2.

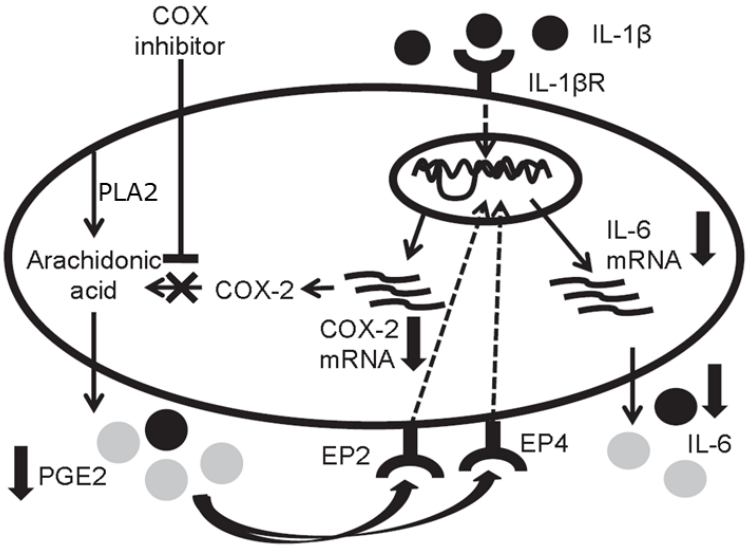

Figure 2: Effect of COX-2 inhibitors on the level of PGE2 and IL-6 after stimulation with interleukin-1β (IL-1β). The presence of COX inhibitors reduce the gene expression of COX-2 resulting in lower PGE2 levels. PGE2 concentration affects IL-6 production through prostaglandin E2 receptors 2 and 4 (EP2, EP4), modified after [20].

Neutrophils and macrophage immune cells produce and respond to the IL-6 concentration and result in the amplification of inflammation related signals or transform an acute to a chronic inflammatory state through repeated macrophage infiltration and repeated damage to the extracellular matrix of the dermis [21,22]. Continuous, low-grade inflammation levels cause tissue damage and are considered as one of the driving forces for intrinsic skin aging and is referred to as caspase-1 mediated inflammation [23,24]. Targeting prostaglandin production by inhibiting COX-2, the rate-limiting enzyme, is one of the options to treat inflammatory skin diseases [8,19].

3.2. Anti-Inflammatory Plant Medicine

Currently, both traditional and non-steroidal anti-inflammatory drugs (NSAIDs) are the commonly prescribed medications that treat inflammatory diseases by targeting COX-2 [25,26]. Evidence from clinical trials suggests that prolonged use of NSAIDs is associated with severe and sometimes life-threatening side effects [27,28]. It is therefore imperative to find alternative therapeutic interventions with

comparable efficacy and fewer side effects. Naturally occurring compounds have been reported to inhibit COX-2, thereby possessing beneficial effects against inflammation, and accompanied cell injury [26,29]. Throughout human civilization, medicinal plants have been used to treat all kinds of inflammatory conditions and their synthetic and semi-synthetic derivatives contribute to most clinically used medicines. The contribution of phytochemical and ethnopharmacological studies play a key role leading to the identification, isolation, characterization and exploration of the mechanisms of action of a variety of natural compounds. However, given the plant extract complexity, the actual efficacy and the relevant active principles for many of the plants in use remain unknown. Substances contributing to the anti-inflammatory effect are regularly merely assumed and, in most cases, unknown or assigned to molecules whose content in the extract is insufficient or not known at all. Consequently, experimental studies to demonstrate the pharmacological properties of these plants and identifying the relevant active compounds are needed. In this context, *Waltheria Indica L.,* a medical plant with reported anti-inflammatory properties was used in this thesis to identify compounds regulating inflammatory immune responses via COX-2 inhibition.

3.3. Waltheria indica Linn

Waltheria indica L., belonging to the Malvaceae family, grows in tropical and subtropical regions of the world. Waltheria is a traditional medicinal plant with anti-inflammatory properties used by indigenous populations in different regions of the world for the treatment of various pathological conditions [30,31]. The biologically active compounds proved to be present in all parts of *Waltheria indica* are reported in roots, stems, or leaves for the treatment against swelling, cough, toothache, sore throat, rheumatism, or complicated ailments such as asthma and inflammatory skin diseases [31-35]. Studies on the properties of *Waltheria indica* showed that its extracts have multiple anti-inflammatory activities, often without attributing the effect to a single molecule. Extracts that exhibit biological activity include various chemical groups such as alkaloids, flavonoids, sterols, terpenes, anthraquinones or carbohydrates [31, 36,37]. Hydroalcoholic extracts of the whole plant of *Waltheria indica* for example strongly inhibited edema at the second phase of carrageenan inflammation in rats [38]. Extracts from leaves generated with hydrophobic solvents such as petrolether or methanol, but also extracts from leafy stems generated with hydrophilic solvents such as water showed dose-related inhibition of acute and chronic inflammation in carrageenan induced edema. The effect is presumed to involve inhibition of histamine, serotonin, bradykinin, prostaglandin, and cyclooxygenase (COX) related-activities [29-31]. Studies aimed at uncovering the anti-inflammatory molecules in Waltheria and have identified three flavonoids (-)-epicatechin, quercetin, tiliroside and two alkaloids waltherione A and C as potential active molecules [30,31,42]. The alkaloids waltherione A and C, obtained from decoction of roots and aerial parts, have been shown to inhibit nuclear factor (NF-κB) [43]. Quercetin and tiliroside showed a dose-dependent inhibition of the production of inflammatory mediators, including nitric oxide (NO), tumor necrosis factor (TNF-alpha), interleukin (IL)-12 and COX-2 [42,44,45]. It was suggested that the observed inhibition of lipoxidase-5 (5-LOX) and phospho-lipase A2 (PLA2) by the hydroalcoholic Waltheria extracts is due to (-)-epicatechin, which in other studies demonstrated COX-2 inhibitory properties [30,31,39,46]. Although these molecules exhibited efficacy in the performed assays, it was not evident from these studies

whether their content in the tested extracts was sufficient or different substances contributed to or were responsible for the observed activity. In addition to the substances isolated from *Waltheria indica*, other classes of compounds have been explored in various plants that exhibit significant COX-2 inhibitory activity and presumably present but not yet identified in *Waltheria indica* [47].

3.4. Phytochemicals and anti-inflammation

Phytochemical is a broad term referring to a wide variety of compounds produced naturally by plants [48]. They are a promising source of anti-inflammatory molecules with numerous reports on the bioactivity of many of these natural products (NPs) with anti-inflammatory potential. However, their optimization and exploration remain limited by the lack of a comprehensive understanding of their effective scaffold structure or biological targets. Based on their chemical structures and characteristics phytochemicals have been classified into phenolics, carbohydrate, terpenoids lipids, and alkaloids including other nitrogen-containing compounds (Figure 3). Within each category, further division based on biogenesis or biosynthetic origin gives rise to different subcategories.

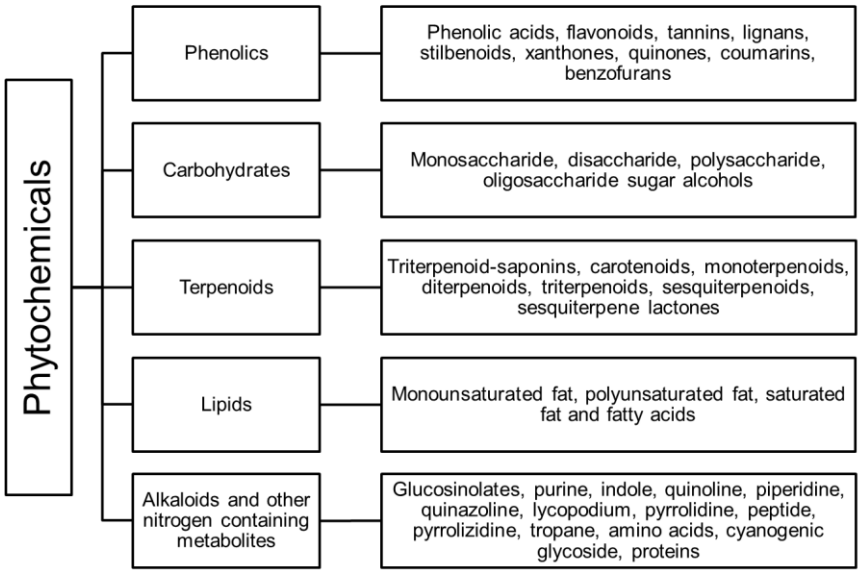

Figure 3: Categorization of phytochemicals into phenolics, carbohydrate, terpenoids lipids, and alkaloids including other nitrogen-containing compounds modified after [49].

A recent study on phytochemicals with reported anti-inflammatory activity showed that terpenoids represent by number the largest group of anti-inflammatory compounds, followed by flavonoids, alkaloids, basic aromatic products and lignans [50]. However, it is not described to what proportion the anti-inflammatory properties are the result of COX-2 inhibition, as the study did not address this aspect. In a comprehensive review of phytochemicals with COX-inhibiting effects, flavonoids, alkaloids, terpenoids, saponins and fatty acids were found to be among the most prominent representatives without stating a specific distribution among them [47]. Phytochemical studies on *Waltheria Indica L*. leaves are currently limited to qualitative statements. To enable the identification of compounds in *Waltheria Indica L*. regulating inflammatory immune responses via COX-2 inhibition, the quantification of potential active phytochemicals is essential. In this context the content of total phenols, alkaloids,

steroidal-saponins, triterpenoid-saponins and the flavonoid tiliroside were investigated in *Waltheria Indica* leaf extracts.

3.4.1. Phenols

Phenolic compounds contain an aromatic ring with one or more hydroxyl groups and are classified into subgroups as flavonoids, tannins, phenolic acids and miscellaneous group (lignans, stilbenoids, coumarins, etc.) [51]. Flavonoids are polyphenols and one of the largest groups of naturally occurring phenolic compounds [52]. They have a general structure of a 15-carbon skeleton, consisting of two phenyl rings (A and B) and a heterocyclic ring C, containing the embedded oxygen. The structural diversity of flavonoids is due to the multitude of substitution patterns on rings A and B and are further divided into subclasses: flavanones, flavones, flavanonols, flavonols, flavandiools, flavananols and anthocyanidins [53]. Examples of phenolic phytochemicals are shown in Figure 4.

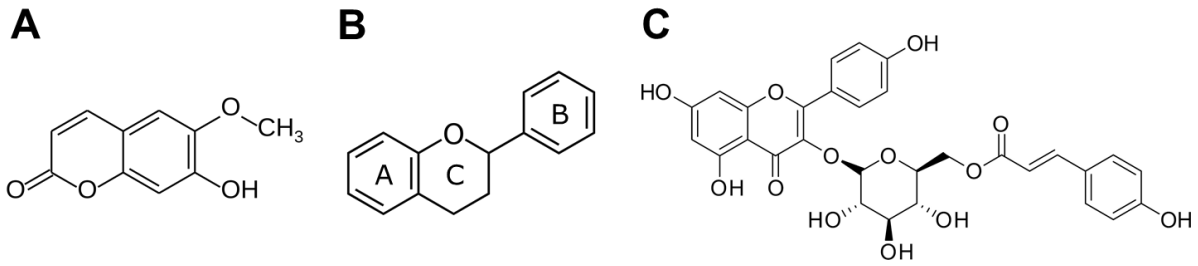

Figure 4: Example of phenolic chemical structures. A: Scopoletin, a coumarin with reported COX-2 inhibition; B: flavonoid backbone structure; C: Tiliroside, a flavonoid identified in *Waltheria indica*.

Phenolic compounds modulate a broad spectrum of inflammatory regulatory key points, with their antiphlogistic action deriving from simultaneous actions on different molecular targets. Several pathways mediate the antiphlogistic action of phenols: their antioxidant and pro-oxidant effects, inhibition of the expression of inflammation-related genes, interaction with signaling pathways, and the interaction with pro-inflammatory proteins, including the inhibition of specific enzymes such as COX-2 [54,55]. Docking studies with several flavonoids indicate that some flavonols and flavones containing a 2,3-double bond act as preferential inhibitors of COX-2 [56]. Further studies suggested that flavanones selectively inhibit COX-2 by forming two hydrogen bonds (Tyr-371 and Ser-516) in the active site of COX-2 [57]. Given the vast diversity of phenolic compounds, no definite correlation between the COX-2 inhibitory effects and their chemical structure is described yet.

Currently, no consistent analytical procedures for the quantification of the individual phenolic subgroups are described [58-60]. Investigations on quantification of different phenolic compounds showed that most of them were reactive in the presence of the Folin-Ciocalteu reagent, and the corresponding intensity of absorption was proportional to their concentration in plant extracts [61]. For a reliable correlation between concentration of phenolic compounds and their activity, the total phenol content was determined using the Folin-Ciocalteu method in this thesis.

One of the known phenolic compounds in *Waltheria Indica* L. is the flavonoid tiliroside. Tiliroside is an antioxidant exhibiting a dose-dependent inhibition of the production of inflammatory mediators, including

nitric oxide (NO), tumor necrosis factor (TNFα) and interleukin (IL)-12 [42,44]. The molecular anti-inflammatory effect involves the down regulation the protein expression levels of iNOS and COX-2 and the reduction of mitogen-activated protein kinase (MAPK) signaling [62]. *In vivo* studies showed that topical application of tiliroside significantly reduced UV-induced erythema compared to placebo [63]. However, there have been no reports on the content of tiliroside in *Waltheria indica* and whether it modulates the COX-2 inhibition observed with extracts generated from *Waltheria indica*. In view of the observed anti-inflammatory properties, especially COX-2 inhibition, of tiliroside, this study aims to identify optimal extraction conditions for *Waltheria indica* to maximize the content of tiliroside in the extracts to achieve a maximum potent COX-2 inhibitory activity.

3.4.2. Alkaloids

The alkaloids comprise one of the largest class of secondary plant substances. They fulfil a remarkable range of pharmacological activities including significant anti-inflammatory properties [64,65]. Alkaloids are nitrogen-containing compounds with predominantly heterocyclic nitrogen, which occur as secondary and tertiary amines, as amides, amine oxides and quaternary ammonium bases. Which nitrogen-containing natural substances fall into the substance class of alkaloids is subject to a certain randomness, whereby the boundary is drawn differently depending on the author and the state of research. A wide variety of alkaloids with potent COX-2 inhibitory properties have been characterized to date [47]. Among the common classes for which a COX-2 inhibitory activity has been observed include quinoline alkaloids, indole alkaloids, piperidine alkaloids, and quinazoline alkaloids [66]. Figure 5 shows examples of alkaloid structures.

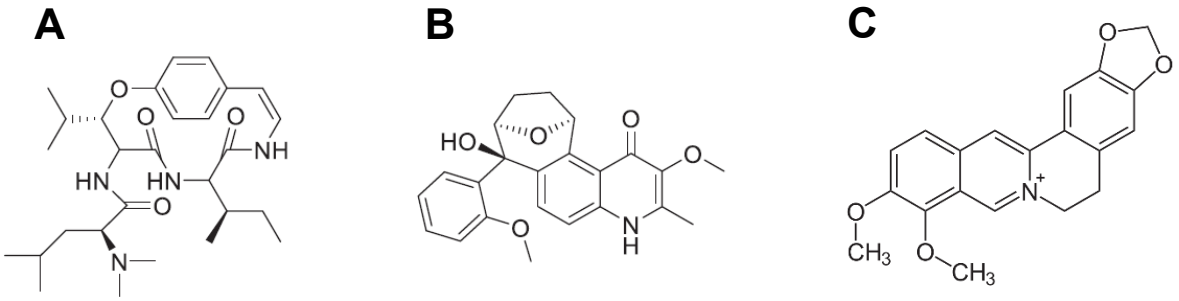

Figure 5: Examples for alkaloid molecule structures. Alkaloids identified in Waltheria Indica, A: Adouetin X and B: Waltheriones A. C: Berberine, an alkaloid present in plants with reported COX-2 inhibitory activity.

Chemical investigation of *Waltheria indica* extracts led to the isolation and characterization of more than 20 alkaloids in the last 10 years [67-70]. Two of them waltherione A and C, have been demonstrated to expose anti-inflammatory activity via inhibiting NF-κB [43]. To date, there are no studies on the content of alkaloids in *Waltheria indica* leaves and whether they contribute to the COX-2 inhibitory effect. Given that alkaloids have been detected in *Waltheria indica* and belong to the substance classes that potentially contribute to COX-2 inhibition, their total content in the extracts was investigated in this thesis to determine whether they contribute to the observed COX-2 activity. The quantification of total alkaloids was performed based on the precipitation of alkaloids by Dragendorff reagent (DR) followed by the formation of a yellow bismuth complex in nitric acid medium with thiourea [71].

3.4.3. Saponins

Saponins are composed of polycyclic aglycones attached to one or more sugar side chains and are ubiquitously distributed in the plant kingdom [72]. The complexity of saponin structures is based on the different glycan moieties that are attached to the triterpenoid (triterpenoid-saponins) or steroidal aglycones (steroidal-saponins) [73]. The foaming and emulsifying properties of saponins result from their amphipathic nature, i.e., the hydrophobic aglycone skeleton and the hydrophilic glycan moiety. Saponins exhibit an extensive range of bioactivities including antimicrobial, antifungal, allelopathic, insecticidal, pesticidal, molluscicidal, hypocholesterolaemic, anticarcinogenic, antiviral and anti-inflammatory activities [74]. The literature contains a large number of reports on saponins isolated from plants with COX-2 inhibitory activity [47,75-77]. Given that saponins are poorly absorbed as glycosides in the intestine, it is assumed that any pharmacological activity observed would be as result of their aglycones [78]. Figure 6 shows example molecules for triterpenoid- and steroidal-saponin.

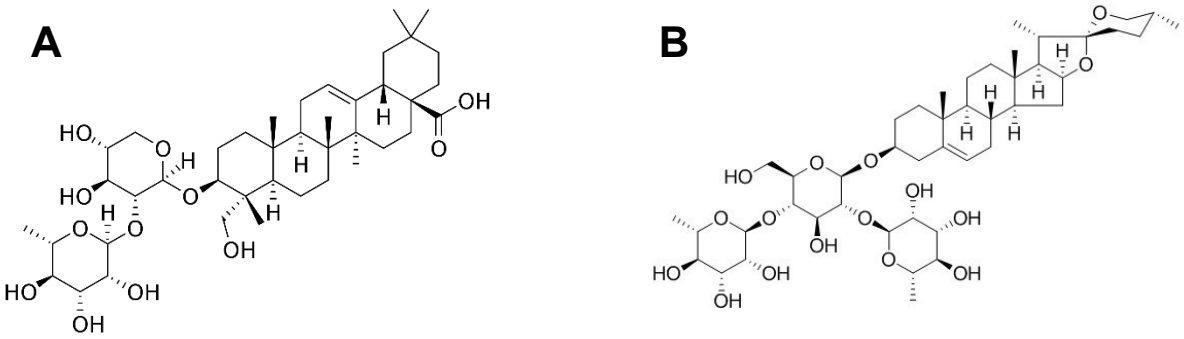

Figure 6: Examples for saponin molecule structures. A: α-hederin, a pentacyclic triterpenoid-saponin; B: dioscin, a steroidal-saponin.

Recently several triterpenoids including betulinic acid, β acetoxy-27-trans-caffeoyloxyolean-12-en-28-oicacid methyl ester and β-acetoxy-27-cis-caffeoyloxyolean-12-en-28-oic acid methyl ester have been isolated from the aerial parts of *Waltheria indica* without specifying the content in the extract [43,79]. Although there are several studies that have demonstrated the presence of saponins in *Waltheria indica*, no studies on the saponin content have been reported yet. To determine whether saponins contribute to the observed COX-2 activity, the total content in the extracts was quantified in this study. Different analytical approaches were employed to quantify triterpenoid- and steroidal-saponins individually and the influence of extraction parameters.

3.5. Plant extraction

The first step toward isolating and identifying the specific compounds responsible for biological activities associated with a plant or a plant extract is their extraction from the plant material. The extraction of an active ingredient is influenced by multiple factors, including characteristics of the raw material matrix, extraction technique employed, solvent type, temperature used or the solvent to plant material ratio [80]. Depending on the group of substances to be isolated and their physical properties, the extraction method is adapted. No standardized extraction method is available for all substances. This has led to the development of different extraction techniques, varying in cost and level of complexity, for the isolation

or enrichment of phytochemicals. Regardless of the extraction technique employed, the solubility of the target compounds in the solvent used for the extraction is the most relevant aspect of the entire extraction process and the most influential aspect in method optimization [81]. With respect to the solvent polarity, different phytochemicals are preferentially extracted as summarized in Table 1.

Table 1: Overview of phytochemicals extracted with different solvents [82]. [1]part of the uncharacterized phytochemicals; [2]part of the total-phenol concentration; [3]quantification of triterpenoid-saponin types; [4]quantified separately as triterpenoid- and steroidal saponins; [5]quantified as total-alkaloid content.

Solvent	Water	Methanol	Ethanol	Ethyl acetate
Relative polarity	1	0.76	0.65	0.23
Phytochemicals	Starches[1]	Lactones[1]	Tannins[2]	Terpenoids[3]
	Phenols[2]	Tannins[2]	Polyphenols[2]	Saponins[4]
	Terpenoids[3]	Polyphenols[2]	Terpenoids[3]	Flavonoids[2]
	Polypeptides[2]	Terpenoids[3]	Alkaloids[5]	
	Saponins[4]	Alkaloids[5]	Saponins[4]	
		Saponins[4]		

The characteristics of the raw material matrix are important as they provide the potential locations for the target compounds in the matrix. It is assumed that the compound present in natural products may be eighter dissolved in the pore of the matrix, adsorbed on the surface of the matrix, adsorbed in a pore, chemically bounded to the matrix, or dissolved in the bulk solution [83] . Depending on the location in the matrix, the desired substance will have varying degrees of extractability. It has been proposed as schematically shown in Figure 7 that the solvent extraction mechanism involves following steps:

1) Solvent is transferred from the fluid phase to the solid surface of the plant material and pervades it.

2) The solvent enters the plant matrix by molecular diffusion.

3) Soluble compounds are solubilized by desorption from matrix and solvation into the extraction solvent.

4) Solution containing the solutes returns to the surface of the plant material by molecular diffusion.

5) The solution is transferred from the solid plant material surface to the bulk extraction solvent by natural or forced convection.

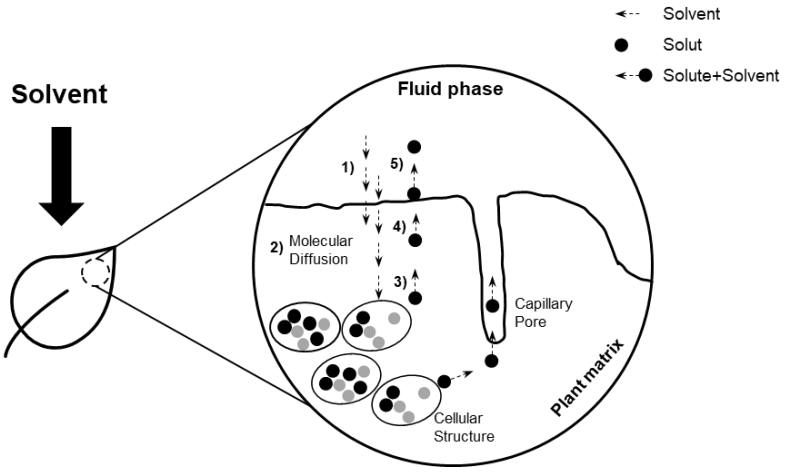

Figure 7: Schematic representation of the extraction mechanism of plant material using liquid solvent: 1) solvent pervades the solid surface of the plant material matrix; 2) solvent enters the plant matrix by molecular diffusion; 3) soluble compounds are solubilized into the extraction solvent; 4) Solution containing the solutes returns to the surface of the plant material by molecular diffusion; 5) solution is transferred from plant material surface to the bulk extraction solvent, modified after [84].

The extraction of a phytochemical component (X) from plant matrix (A) to the extraction solvent phase (B) begins when the two phases come into contact. The distribution of X between the immiscible phases occurs as soon as it can be transferred from phase A to phase B and back from phase B to phase A. The solubilization limit is the equilibrium concentration between the phases. However, in practical applications of the extraction process the equilibrium is almost never reached because the number of soluble compounds (X) is usually small compared to the amount of solvent available, resulting in a diluted solution in phase B. Therefore, instead of waiting for equilibrium to be reached, usually after a certain amount of component X is transferred to phase B, the process is stopped. Underling that the mass transfer kinetics is important in the extraction process.

The dissolution rate of the solute X into the fluid phase B is controlled by the mass transfer rate of X that moves from the solid matrix (A) into the bulk solvent (B). The solute transfer inside the plant matrix occurs due to a concentration gradient in A, which is strictly controlled by molecular diffusion. The equation that describes the diffusion phenomenon is based on Fick's Law (**equation 1**):

$$\frac{\dot{M}_X}{A_T} = -D_{XA}\frac{dC_X}{dz}$$

Equation 1: Fick's law with M_X = the mass transfer rate of the solute X, A_T = the mass transfer area, represented by the solid–fluid interface, D_{XA} = diffusion coefficient of the solute X into the solid phase A, C_X = the gradient concentration of X inside the solid particle, and z = distance measured from the particle interior.

At the surface of the solid plant matrix particles the solute transfer occurs due to diffusion and convection simultaneously. In this step, the mass transfer rate can be expressed with **equation 2**.

$$\dot{M}_X = k_B A_T (C_{XAI} - C_{XB})$$

Equation 2: Mass transfer as function of k_B = the individual mass transfer coefficient of the fluid phase (B), C_{XAI} = the concentration of X in the solution located at the solid–fluid interface, and C_{XB} = the concentration of X in the bulk solution.

In extraction processes the mass transfer rate is not constant and follows a kinetic consisting of three distinct phases: constant extraction rate period (CER), falling extraction rate period (FER), and diffusion-controlled period (DC) [85]. In the CER period, the readily accessible solute that surrounds the plant matrix particles is removed at an approximately constant rate (Figure 8). In this step the mass transfer resistance is mainly in the stagnant film surrounding the plant matrix particle with the main mechanism responsible for the mass transfer being convection. In the FER period, gaps appear in the solute superficial layer covering the solid plant matrix and mass transfer resistance in the solid–fluid interface occurs. In this step, the extraction rate decreases due to the reduction of the effective mass transfer area, and diffusion becomes more important. In the DC period the readily accessible solute layer is depleted, and the extraction rate being determined exclusively by the diffusion rate of the solvent into the solid plant matrix particles and of the solvent and solute from the plant matrix to the bulk extraction solvent.

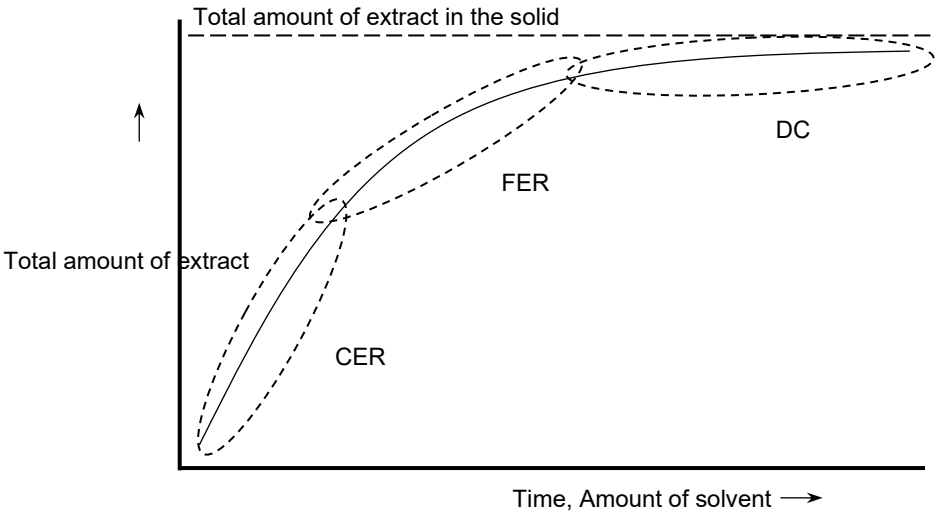

Figure 8: Typical kinetic curve observed for the extraction of natural products consisting of three distinct phases: constant extraction rate period (CER), falling extraction rate period (FER), and diffusion-controlled period (DC); adapted from [85].

Another challenge of the extraction process is that the target compounds are potentially not readily accessible and interact with other components of the raw material such as proteins or carbohydrates [84,86]. Here, the intermolecular interactions between these molecules must be disrupted in order to prevent the formation of new intermolecular interactions between the solute and the solvent. This can be realized by providing sufficient energy to break the linkages to effectively extract the compounds that interact with the solid matrix with the amount of energy spent in the process affecting the extraction efficiency.

The classic and commonly used solvent-based methods include maceration, percolation, Soxhlet extraction or steam distillation [80]. While these relatively simple techniques can be effective and economical on small scale, they are time and solvent consuming with reproducibility of results not always being guaranteed, making them less suited for screening experiments. The Green Chemistry movement has led the change toward sustainable and environmentally benign extraction techniques such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) [87-89].

In order to ensure constant conditions during extraction and high reproducibility of the results, the plant extraction in this thesis was carried out using the ASE technique. The system utilized is designed to perform automated subcritical solid-liquid extractions while controlling and maintaining temperature and pressure for each extraction. The application of high pressure allows extractions at temperatures above the boiling point of most solvents, as the solvent remains in the liquid state of aggregation. The use of elevated temperature increases the capacity of solvents to solubilize analytes, i.e., solubility of any solute in a solvent increase with the increase of solvent temperature. Moreover, higher temperature reduces viscosity, that the solvent penetrates the pores of the matrix more easily resulting in faster diffusion rates. Additionally, increased temperature disrupts the strong solute–matrix interactions caused by van der Waals forces, hydrogen bonding, and dipole attractions and remove the solute from matrix [90]. Operating at elevated pressure accelerates the extraction process as the pressurized solvent is forced into the pores of the sample matrix, resulting in more close contact with the analytes in

those areas. The utilization of an automated extraction system in combination of elevated temperature and pressure allows the extraction in the range of minutes, resulting in high efficiency and reproducibility with lower solvent consumption levels [91-93].

3.6. Centrifugal partition chromatography

In the framework of this thesis, the identification of the molecules responsible for the COX-2 inhibitory activity of the extracts besides the tiliroside will be investigated. The utilization of centrifugal partition chromatography (CPC) is aimed to simplify the extract matrix and to allow a targeted identification of the active fractions with subsequent characterization of the compounds involved.

CPC is based on the use of two immiscible liquid phases that differ in density. In contrast to solid-liquid partition chromatography, such as HPLC, the stationary phase is a liquid, which is immobilized by an applied centrifugal force of a rotor in extraction chambers connected in series [94,95]. The centrifugal field is generated by rotation around an axis. The mobile phase is pumped through the extraction chambers and flows through the immobilized stationary phase. An interaction of the liquids, as shown schematically in Figure 9, takes place in the chambers and not in the intermediate channels.

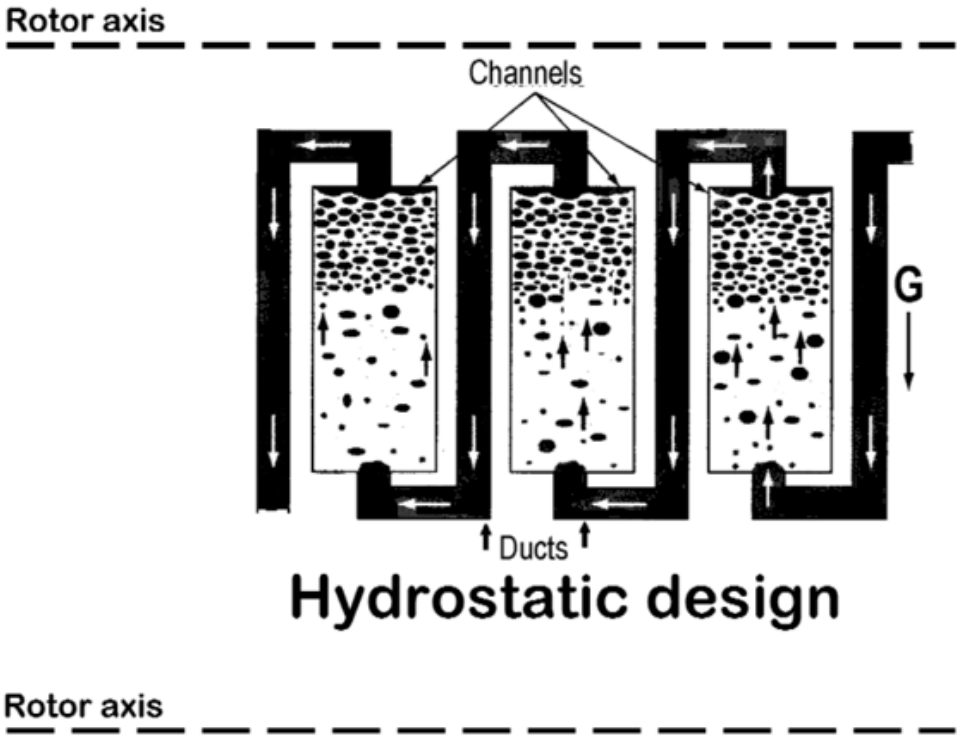

Figure 9: Schematic illustration of the fluid movement in the extraction chambers and ducts in a CPC system [95]. The stationary phase has a higher density. The mobile, lighter phase is transported through the extraction chambers.

During this process, part of the stationary phase is displaced from the extraction chambers and a fluid-dynamic equilibrium is established between the mobile and the stationary phase. After the equilibrium has been set, only the mobile phase leaves the CPC system. The sample is injected manually after the fluid dynamic equilibrium has been set in a CPC system.

In the separation or purification of natural substances, the use of a liquid-liquid separation system has several advantages over the traditionally used solid-liquid chromatography methods. Compared to solid-

liquid chromatography, CPC has a relatively large volume of stationary phase. This leads to a higher loading capacity of the system, which results in a higher productivity in the purification of compounds. Furthermore, a sample does not have to be completely dissolved for separation by CPC. This allows the injection of higher concentrations of the mixture to be separated. Furthermore, a filtration step of raw mixtures, as occurs in the extraction of natural products, can be omitted. Given that no irreversible adsorption occurs on a solid stationary phase the recovery rate of the components after separation is greater than 90 %. Furthermore, there is no reaction of the sample components with the stationary phase. This phenomenon can certainly occur with unstable compounds in solid-liquid chromatography [96-98].

In principle, each of the two liquid phases during the CPC separation can be used as a mobile or stationary phase. There are two main operating modes in the CPC, *Ascending* and *Descending*, which can be explained by the difference in density of the two liquid phases. In *Ascending mode,* the lighter phase, as a result of the lower density, is used as the mobile phase. Here, the mobile phase is conveyed from the outer end of the extraction chambers, in relation to the central axis of rotation, to the inner end, against the direction of the centrifugal vector field. The mobile phase flows through the stationary phase, which settles at the outer end of the extraction chambers due to its higher density [99]. This mode of operation is shown schematically in Figure 9. The mobile phase flows through the stationary phase from the lower to the upper end of the extraction chamber. In *Descending* mode, the heavier phase is used as the mobile phase. The mobile phase is conveyed from the inner end of the extraction chamber to the outer end, with the direction of the centrifugal vector field. The stationary phase settles at the inner end of the extraction chamber due to its lower density and the mobile phase flows through.

In addition the operating mode can be switched during the ongoing separation sequence, which led to the development of further operating modes such as "elution-extrusion" [100,101]. Here, an elution of compounds with the mobile phase takes place first. In the second step, partially separated hydrophobic compounds, which are still in the stationary phase, are extruded with stationary phase. In general, two processes take place during a compound separation process in a CPC plant. There is an elution process of the substances from one phase to the other, and there is a retention process of the substances in the stationary phase. The chromatographic separation of compounds is thereby invariably based on different partition coefficients of each compound between the stationary and mobile phases. In order to realize the separation of the extracts into distinct fractions, which in sum depict all components of the extract, a $2V_C$ Elution-Extrusion method was applied.

In short, the method is performed in two steps, each step using one column volume of each phase of the biphasic liquid system. Step one is the classical elution step lasting for exactly one column volume. The solute retention volumes (V_R) are linked to their partition coefficients (K_D) by equation 3:

$$V_R = V_M + K_D V_S$$

Equation 3: Solute retention volume (V_R) as function of mobile phase volume (V_M), stationary phase volume (V_S) and the partition coefficients (K_D).

The column volume (V_C) is the sum of mobile phase and stationary phase volume (equation 4).

$$V_C = V_M + V_S$$

Equation 4: Column volume (V_C) as function of mobile phase volume (V_M) and stationary phase volume (V_S).

Consequently, for any CPC column configuration, the solute that partitions equally between the two-liquid phase ($K_D = 1$) is eluted with a V_C retention volume.

During step one, the elution step, solutes with low partition coefficients $0 \leq K_D \leq 1 + V_M/V_S$ are eluted with retention volumes:

$$V_R \leq V_C + V_M$$

After eluting one column volume of mobile phase, step two is started by changing the entering liquid phase for the other phase, which was the stationary phase during step one. The normal chromatographic process goes on as long as the V_M volume of mobile phase is not extruded out of the column.

During step two, the extrusion step, all remaining solutes with partition coefficients $1 + V_M/V_S \leq K_D \leq \infty$ are eluted with retention volumes:

$$V_C + V_M \leq V_R \leq 2V_C$$

After the stationary phase from step one reaches the column outlet all compounds contained in the column are extruded. When a column volume of stationary phase has been passed in the column, there is no compound that could possibly remain in the CPC column.

The selection of a suitable solvent system for efficient separation of complex mixtures such as plant extracts is of fundamental importance in CPC processes [102]. A variety of solvent combinations are used to create a biphasic solvent system resulting in numerous solvent systems with different polarity and selectivity combinations. To speed up the selection of suitable systems, solvent systems were grouped into families. These families consist of the same solvents that differ in their mixing ratio, thereby providing a methodical approach for finding of particular solvent system that predicts suitable partition coefficients for the target compounds.

For the separation of known molecules in the extract, the partition coefficients of the individual compounds of interest can be determined experimentally in different solvent systems and mathematical equations applied for their optimal separation. However, in the context of this work, a separation is performed in order to obtain extract fractions composed of unknown compounds. The simplification of the extract matrix is intended to enable a targeted identification of active fractions and subsequently the characterization of the compounds involved. To determine appropriate solvent systems for the CPC fractionation, a systematic screening strategy was adapted as described in the literature, using different rations of heptane, ethyl acetate, methanol and water to generate a two-phase solvent system from the Arizona solvent family (AZ) [103]. The Arizona solvent family consists of 21 different solvent combinations, whereby each letter of the alphabet from A to Z represents a defined composition of four solvents [104]. The screening strategy is illustrated in Figure 10.

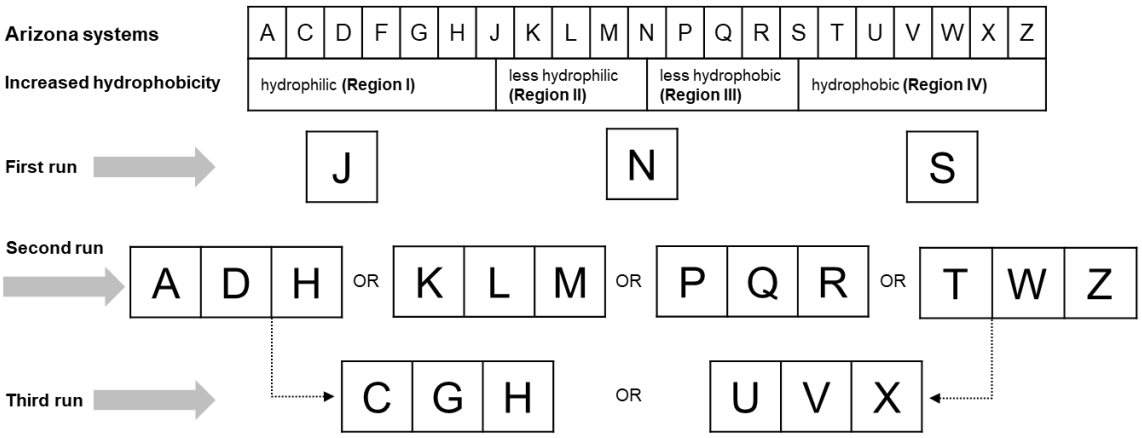

Figure 10: Screening strategy with different biphasic liquid compositions of the Arizona liquid system (AZ) adapted from [103].

The AZ liquid system forms a scale of compositions with polarity decreasing from the A binary system to the less polar Z system. The solvent proportions in the AZ system are symmetrical toward the central N composition with an equal amount of heptane, ethyl acetate, methanol, and water and therefore as the middle polarity starting point. The A-M polar compositions are further divided in two regions: region I, hydrophilic, from A to H and region II, less hydrophilic, from K to N. Similarly, the N-Z less polar compositions are divided in region III, less hydrophobic, from N to S and region IV, hydrophobic, from T to Z. In the first run, the results of the solvents compositions J, N and S will be studied and, according to the results decided in which polarity direction the next step should be carried out.

3.7. COX-2 inhibition prediction model

In vitro testing systems that investigate the activity of COX-2 inhibitors involve eighter the use of cell lines cultured in the presence of the test compound, isolated human blood cells or isolated enzyme preparations. Assays that are based on the cultivation of cells or separation of cells from blood have in common that they are time-consuming, cost-intensive and are less applicable for routine testing. Enzymatic assays on the other hand do not resemble pathophysiological conditions as parameters such as cell–cell interactions and plasma binding of the drug are neglected. However, using enzymatic assays to investigate COX-2 inhibitory properties avoids disturbing endogenous influences and receives exact information about the actual and sole impact of the compounds on the enzyme. The use of established COX-2 screening tests allows the testing of a large number of samples in a short time and at a manageable cost, which makes them suitable for screening investigations for the activity prediction. In the framework of this thesis, COX-2 inhibitory activity of *Waltheria indica* extracts, extract fractions and single compounds, were examined utilizing an enzyme-based assay based on the fluorometric detection of the probe-marked product of COX-2 reaction.

In order to predict the activity of compounds, structure-activity relationship (SAR) models have been established with the objective of finding relationships between the chemical structure (or structure-related properties) and the biological activity (or target property) of the compounds under investigation. The central axiom of SAR is that the activity of molecules is reflected in their structure with similar molecules having similar activities [105,106]. While the biological effects of a new chemical compounds

can often be predicted from its molecular structure using data about other similar compounds, the prediction of the activity of complex mixtures such as plant extracts still remains a major challenge.

The attempt to develop a prediction model for the COX-2 inhibitory activity utilizing *Waltheria indica* extracts aims to establish relationships between the individual phytochemicals and their contribution to the activity. The development of the model will be examined under the consideration of the sum of individual phytochemicals in the extracts as the predictor variable for the COX-2 activity. While for extraction with one solvent at different temperatures it can be assumed that the same phytochemicals are contained in the extracts in different compositions, the objective of this study was to take a further step and develop a model for the prediction of COX-2 inhibitory activity based on extracts generated with different solvents. The use of different solvents has the consequence that further phytochemicals are extracted in different compositions (Table 1). The increase in complexity is intended to enable a more robust prediction of the activity of the extracts independent of the solvents used.

3.8. References

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4. Aims of the thesis

During the course of this doctorate project, the following questions and objectives were investigated:

- How do the polarity and the temperature of the extractant during the extraction of *Waltheria Indica* leaves affect the tiliroside extraction rate and which parameters result in the maximization of its content in the extract?

- Develop a correlation between the COX-2 inhibitory activity of the extracts based on their tiliroside content

- How is the content and composition of the phytochemicals in the extract affected by the extraction parameters and is a correlation to the COX-2 inhibitory activity of the extracts discernible?

- Identify compounds with COX-2 inhibitory activity and evaluate their contribution to the activity in the extract established by extract fractionation using an activity-guided approach.

- Define concentrations of metabolite classes in extracts and define how they contribute to COX-2 inhibitory activity.

- Based on potential correlations built a mathematical model on phytochemical classes how they contribute to the COX-2 activity.

5.1

Identification of plant metabolite classes from *Waltheria Indica L.* extracts regulating inflammatory immune responses *via* COX-2 inhibition

Identification of plant metabolite classes from *Waltheria Indica L.* extracts regulating inflammatory immune responses *via* COX-2 inhibition

Michael Termer[1], Christophe Carola[3], Andrew Salazar[3], Cornelia M. Keck[1],

Juergen Hemberger[2], Joerg von Hagen[2,3]

[1] Department of Pharmaceutics and Biopharmaceutics, Philipps-University of Marburg, Marburg, Germany

[2] Department of Life Science Engineering, Institute for Biochemical Engineering & Analytics, University of Applied Sciences, Giessen, Germany

[3] Merck KGaA, BU Performance Materials, Darmstadt, Germany

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Abstract

Ethnopharmacological relevance: Waltheria Indica L. is traditionally used in Africa, South America and Hawaii to treat pain, anemia, diarrhea, epilepsy and inflammatory related diseases.

Aim of the study: This study aimed to identify extraction parameters to maximize tiliroside yield and to quantitative secondary metabolite composition of Waltheria Indica under various extraction conditions. The extracts were tested for COX-2 inhibition and their activity correlated with the type and quantity of the secondary metabolites. Insight was gained about how extraction parameters influence the extract composition and thus the COX-2 enzymatic inhibitory activity.

Materials and methods: Powdered leaves of Waltheria Indica were extracted using water, methanol, ethyl acetate and ethanol at different temperatures. Tiliroside was identified by HPLC-HRMS n and quantified using a tiliroside standard. The compound groups of the secondary metabolites were quantified by spectrometric methods. Inhibitory potential of different Waltheria extracts against the COX-2 enzyme was determined using a fluorometric COX-2 inhibition assay.

Results: The molecule, tiliroside, exhibited a COX-2 inhibition of 10.4% starting at a concentration of 15 µM and increased in a dose dependent manner up to 51.2% at 150 µM. The ethanolic extract at 30 ◦C and the ethyl acetate extract at 90 ◦C inhibited COX-2 with 37.7% and 38.9%, while the methanolic and aqueous extract showed a lower inhibition of 21.9% and 9.2% respectively. The results concerning phenol, alkaloid and tiliroside concentration in the extracts showed no dependence on COX-2 inhibition. The extracts demonstrated a direct correlation of COX-2 inhibitory activity with their triterpenoid-/steroidal-saponin concentration. COX-2 inhibition increased linearly with the concentration of the saponins.

Conclusion: The data suggest that Waltheria Indica extracts inhibit the key inflammatory enzyme, COX-2, as a function of triterpenoid- and steroidal-saponin concentration and support the known efficacy of extracted Waltheria Indica leaves as a traditional treatment against inflammation related diseases.

1. INTRODUCTION

Waltheria Indica L., belonging to the Malvaceae family, grows in tropical and subtropical regions of the world. It is one of the most widely used traditional medicinal plants with anti-inflammatory properties with reports supporting the use of roots, stems or leaves for the treatment of cough, sore throat, congestion or complicated ailments such as asthma and inflammatory skin diseases **(Zongo et al., 2013)**.

Hydroalcoholic extracts of *Waltheria Indica* demonstrate inhibition of edema in rat at the second phase of carrageenan induced inflammation as well as inhibiting the expression of key inflammatory cytokines and cytokine receptors, thereby effectively reducing activities of multiple pro-inflammatory signaling pathways and have the potential to modulate the immune response and cure or suppress the onsets of inflammatory diseases **(Vedavathy and Rao, 1995; Laczko et al., 2019)**. The biologically active compounds proved to be present in all parts of *Waltheria Indica* comprise different chemical groups including alkaloids, flavonoids, sterols, terpenes, anthraquinones and carbohydrates (**Borokini and Omotayo, 2012; Cretton et al., 2015**, **Zongo et al., 2013**). Studies focused on the anti-inflammatory properties of Waltheria have identified three well-known flavonoids: (-)-epicatechin, quercetin, and tiliroside (**Rao et al., 2005; Zongo et al., 2013**). Tiliroside is an antioxidant exhibiting a dose-dependent inhibition of the production of inflammatory mediators, including nitric oxide (NO), tumor necrosis factor (TNFα) and interleukin (IL)-12 (**Grochowski et al., 2018**; **Rao et al., 2005**). The molecular anti-inflammatory effect involves the down regulation the protein expression levels of iNOS and COX-2 and the reduction of MAPK signaling (**Jin et al., 2016**). *In vivo* studies showed that topical application of tiliroside significantly reduced UV-induced erythema compared to placebo (**Epstein, 2010**).

Although flavonoids, such as tiliroside, exhibit anti-inflammatory activity, they do not necessarily represent the major group of compounds by mass in the plant extract. Their biological efficacy is rather the result of additive or synergistic effects of multiple substances or biological enhancement due to the presence of substance classes in high concentrations (**Pagare, 2018; Caesar and Cezh, 2019)**. Reports from 665 natural substances revealed that terpenoids represent the largest group of anti-inflammatory compounds, followed by flavonoids, alkaloids, basic aromatic products and lignans **(Zhang et al., 2019)**. The determination of the total amount of different secondary metabolite classes might provide insight in identifying or predicting extracts with anti-inflammatory efficacy.

Inflammation is a localized response to various stimuli and is a cyclic, self-stimulating process designed to combat infection or tissue injury (**Jones, 2005; Pasparakis et al., 2014**). Crosstalk between different cellular components of the skin regulates local immune responses to maintain and restore homeostasis (**Di Meglio et al., 2011; Pasparakis et al., 2014**). Dysregulated inflammatory responses contribute to chronic disease progression and inflammatory chronic or auto-immune skin disorders (**Gallin and Snyderman, 1999; Jones, 2005; Pasparakis et al., 2014**).

Three protein families are important modulators of inflammation like nuclear factor kappa B (NF-kB), lipoxygenase (LOX) and cyclooxygenase (COX) (**Greene et al., 2011; Mantovani et al., 2008**). NF-kB activates transcription of several inflammation related enzymes, transcriptions factors, and signaling molecules. Because of its diverse set of gene targets, activation of NF-kB leads to the expression of

LOX or COX (**Schmitz and Ecker, 2008**). These enzymes are part of the arachidonic acid (AA) metabolism, one of the main cellular processes for mediating inflammation (**King, 2007, Hanna and Hafez, 2018**).

The COX-1 and COX-2 enzymes are isozymes that have cyclooxygenase activity. COX-1 is expressed constitutively in most tissues and is involved in homoeostatic processes, such as regulating blood flow or promoting platelet aggregation (**Zarghi and Arfaei, 2011**). In normal human skin, COX-1 is present throughout the epidermis whereas COX-2 localizes mainly in suprabasal keratinocytes (**Leong et al., 1996; Buckman et al., 1998; An et al., 2002**). The pro-inflammatory COX-2 is an inducible isoform mainly produced in inflamed tissues and together with COX-1 responsible for the conversion of AA to prostaglandin G_2 (PGG_2) and further to prostaglandin H_2 (PGH_2). PGH_2 is converted into prostanoids thromboxane A_2 (TXA_2), prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin D_2 (PGD_2), and prostaglandin F_2 (PGF_2) by individual prostaglandin synthase enzymes (**Dannenberg et al., 2001**; **Ricciotti and FitzGerald, 2011; Nørregaard et al., 2015**). Increased PGE_2 levels lead to increased production of proinflammatory cytokines such as interleukin-6 (IL-6) with inflammatory disease activities directly linked to the IL-6 concentration (**Hinson et al., 1996; Kawahara et al., 2015**; **Paquet and Piérard, 1996**). Neutrophils and macrophage immune cells produce and respond to the IL-6 concentration and result in the amplification of inflammation related signals or transform an acute to a chronic inflammatory state through repeated macrophage infiltration and repeated damage to the extracellular matrix of the dermis (**Choy and Rose-John, 2017; Zhuang and Lyga, 2014)**. Continuous, low-grade inflammation levels cause tissue damage and are considered as one of the driving forces for intrinsic skin aging and is referred to as caspase-1 mediated inflammation **(Youm et al., 2013; Mejias et al., 2018**). Targeting the prostaglandin and IL-6 expression through COX-2 inhibition, the rate-limiting enzyme modulating the production, appears to be decisive for the effective control of this immunological process and relevant for the successful treatment of inflammatory skin diseases (**An et al., 2002**; **Desai et al., 2018**).

This underlines that *Waltheria Indica* is interesting for the use against inflammation related diseases. To achieve potent COX-2 inhibitory activity, an extract with the highest possible concentration of tiliroside should be generated. A study identifying optimal extraction conditions for *Waltheria Indica* leaves to maximize the tiliroside content in the extracts is yet not described. Therefore, the aim of this study was to identify extraction parameters to maximize tiliroside yield. Ideally it should be verified that COX-2 inhibitory activity correlates with the tiliroside content. In addition, the influence of extraction parameters on the composition were determined with regard to the content of phenols, alkaloids, triterpenoid- and steroidal-saponins in the obtained excerpt.

2. MATERIAL AND METHODS

2.1 Chemicals

Tiliroside, gallic acid, oleanolic acid, diosgenin as reference standards and Folin-Cioclateu reagent, Dragendorff reagent, bismuth nitrate, sodium sulfide, nitric acid, vanillin, perchloric acid, sulfuric acid, dimethyl sulfoxide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate, potassium iodide, acetic acid, hydrochloric acid, thiourea, anisaldehyde and phosphoric acid were

obtained from Merck KGaA (Darmstadt, Germany). All other solvents and chemicals used were of analytical or HPLC grade. The working solutions were prepared immediately prior to measurement.

2.2 Plant material

Waltheria Indica L. plant was collected from Wageningen University Netherlands and verified as Waltheria Indica by Eurofins Genomics Europe Applied Genomics GmbH. Leaf samples were dried at a constant temperature of 40 °C for 48 hours (Vacutherm, Thermo Scientific) and grounded into a fine powder.

2.3 Plant extract preparation

Plant extraction was performed by accelerated solvent extraction (ASE, Dionex ASE 350 (ThermoFisher Scientific) to assure constant conditions during extraction and good reproducibility of the results (**Benthin et al., 1999; Kellogg et al., 2017**). For all extractions, 100 mL containers were charged with 10 - 12.5 g plant material mixed with diatomaceous earth (60-033854, Thermo Scientific) as a neutral matrix to assure a 1:20 plant/solvent ratio. The ASE extraction was performed with 4 cycles in total, each cycle having a 6 min static time and a 160 s purge time at 1460 psi static pressure. The extraction temperature was 90 °C for all solvents and additionally 30 °C and 150 °C for ethanol extraction. The obtained extracts were filtered through a 0.22 µm membrane (HPF Millex®, Merck Millipore) and evaporated under vacuum at 60 °C. The extract yield (mg/g plant) was calculated using **equation 1,** tiliroside yield extracted from 1 g plant material (mg/g plant) using **equation 2** and plant material required to obtain 1g extract (g/g extract) using **equation 3**.

Extract yield = weight of extract / weight of dried plant material (**equation 1**)

Tiliroside yield = (concentration tiliroside x yield extract) /weight of dried plant material (**equation 2**)

Plant required = (1 / yield extract) x 1000 (**equation 3**)

The solvents were selected on basis of their polarities ranging from very polar for water (1) to non-polar for ethyl acetate (0.23) as reported by **Reichard (2003)** and summarized in **Table 1**.

Table 1: Overview of used solvents with their relative polarity and extraction temperatures.

Sample	W90	M90	E30	E90	E150	EA90
Solvent	Water	Methanol	Ethanol	Ethanol	Ethanol	Ethyl acetate
Relative polarity	1	0.76	0.65	0.65	0.65	0.23
Temperature [°C]	90	90	30	90	150	90

W90, 90 °C water; M90, 90 °C methanol; E30-E90-E150, 30-90-150 °C ethanol; EA90, 90 °C ethyl acetate.

2.4 Identification and quantification of tiliroside

Identification of tiliroside in *Waltheria Indica* extracts were carried out on Vanquish HPLC system coupled with Thermo Fusion™ Orbitrap Tribrid™ mass spectrometer (Thermo Scientific™) with H-ESI interface and positive ionization. Separation was accomplished using C-18 column (Thermo Acclaim™

120, 100 x 4.6 mm, 5 µm) and gradient elution with solvent A: water/acetonitrile/trifluoroacetic acid 95/5/0.125 (v/v/v) and B: water/acetonitrile/trifluoroacetic acid 5/95/0.125 (v/v/v). Gradient started at 7% (v/v) B, ramping up to 28 % over 15 min, ramping up to 56 % over 5 min, further to 95 % over 5 min, held for 3 min and ramping down to 7 % over 8 min. UV detection was performed at 315 nm, 25 °C column temperature, 1.5 ml/min flow speed and 10 µL injection volume. The chromatograms are summarized in the Supplemental Material and Methods section. The HPLC profile of *Waltheria Indica* extracts showed an intense UV peak (R_t = 14.47 min) with a single charge state of MH+ 595.1 g/mol (Supplementary Fig. 1-2). High resolution mass spectrometer fragmentation data confirmed the identification of tiliroside within *Waltheria Indica* extracts (Supplementary Fig. 3).

Quantitative tiliroside analysis were carried out on Hitachi La Chrom Elite HPLC system coupled with diode array detector (DAD) L-2455 (Hitachi La Chrom Elite). Separation was accomplished using RP-18 column (Chromolith®, 4.6 x 100 mm, Merck KGaA) and gradient elution with solvent A: acetonitrile/water 98/2 (v/v) and B: acetonitrile/water/phosphoric acid 98/2/0.1 (v/v/v). Gradient started at 5% (v/v) A, ramping up to 30 % over 10 min, ramping down to 5 % over 3 min and held for 2 min. UV detection was performed at 315 nm, 30 °C column temperature, 2.5 ml/min flow speed and 10 µL injection volume. The tiliroside concentration present in the extracts was calculated from a five data point calibration curve of tiliroside standard (0.0040 – 0.1210 mg/mL). The results were expressed in mg tiliroside per gram of dry extract (mg/g extract).

2.5 COX-2 inhibitory activity

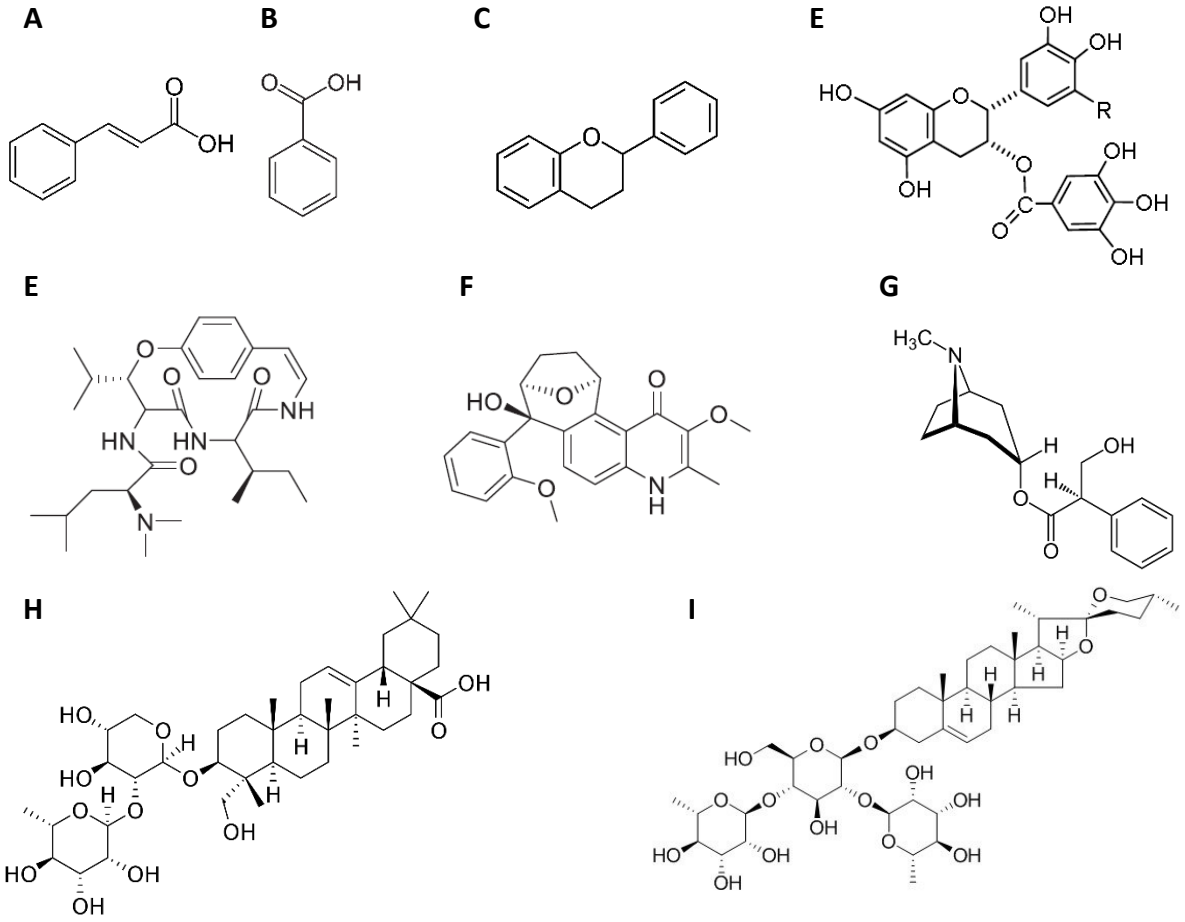
The ability of *Waltheria Indica* extracts to inhibit COX-2, also known as prostaglandin endoperoxide synthase, was determined using the fluorometric COX-2 specific inhibitor screening kit (BioVision, Zurich, Switzerland). The experimental protocol was followed according to the user manual. Fluorescence (Ex/Em = 535/587 nm) of the samples were kinetically measured using a Tecan-Spark multimode microplate reader (Tecan, Switzerland) at 25 °C for 10 min. Appropriate two points (T1 and T2) in the linear range of the plot were chosen, and the corresponding fluorescence values (RFU1 and RFU2) were obtained. The enzymatic assay was applied in the concentration range of 0.1 – 150 µM for tiliroside and 20µg/mL for the extracts dissolved in dimethyl sulfoxide (DMSO). Each sample was analyzed six-fold, while celecoxib (0.5 µM) was used as control and DMSO as blank. The slope for all samples (S), including enzyme control (EC) was calculated by dividing the ∆RFU (RFU2 – RFU1) values by the time ∆T (T2 – T1). Percentage of relative inhibition was calculated using the **equation 4**:

$$\% \text{ Relative inhibition (RI)} = (\text{Slope of EC} - \text{Slope of S}/\text{Slope of EC}) \times 100 \quad (\textbf{equation 4})$$

2.6 Quantitative determination of secondary metabolites

Quantitative determination of secondary metabolites from the group of phenols, alkaloids, triterpenoid- and steroidal-saponins was carried out using spectrophotometric methods. Overview of phenolic (**A**, **B**, **C** and **D**), alkaloid (**E**, **F** and **G**), triterpenoid- and steroidal-saponins (**H** and **I**) basic structures and example compounds quantified as secondary metabolites is illustrated in **Figure 1**.

Figure 1: Overview of core molecular structures and exemplary compounds quantified as secondary metabolites. Structures derived from phenolic acid: cinnamic acids (A) and benzoic acids (B); flavonoid backbone structure (C); hydrolysable tannins (D) epigallocatechin gallate (R=OH) and epicatechin gallate (R=H). Examples for alkaloid molecule structures: alkaloids identified in Waltheria Indica (**Cretton et al., 2015**), Adouetin X (E) and Waltheriones A (F); atropine (G). Examples for saponin molecule structures: α-hederin (H), a pentacyclic triterpenoid-saponin; dioscin (I), a steroidal-saponin.



2.6.1 Phenol quantification

Phenolic compounds contain an aromatic ring with one or more hydroxyl groups and are classified into subgroups as flavonoids, tannins, phenolic acids and miscellaneous group (lignans, stilbenoids, coumarins, etc.) (**Cai et al., 2004; Tsao, 2010; Lu and Luthria, 2014**). The flavonoid analysis and the inter-comparison of data demonstrate that there is no single analytical procedure for an adequate quantification of total flavonoid content in unknown samples and various flavonoid types require a different analytical technique (**Bloor, 2001; Mammen and Daniel, 2012; Julkunen-Tiito et al., 2014**). Recent studies on quantification of flavonoid subgroups showed that the majority is reactive toward Folin-Ciocalteu reagent (primary used for total-phenolics quantification), and the corresponding intensity of absorption was proportional to the concentration of flavonoids **(Ho et al. 2012)**.

Analytical methods for the quantification of tannins are based on precipitation using proteins or polymers such as methylcellulose, polyvinylpyrrolidone, polyvinylpolypyrrolidone, polyethylene glycol and calculating the tannin amount by subtracting the total phenolics amount before and after precipitation

using *e.g.* Folin-Cioclateu reagent **(Sarneckis et al., 2006; Silanikove et al., 1996; Galvão et al., 2018; Makkar et al., 1995).** Studies have shown that the precipitation reagents are not specific for tannins and adsorb flavonoids and other phenolic acids (**Makkar et al., 1995; Mercurio and Smith, 2008; Mitchell et al., 2005**). For a reliable correlation between concentration of phenolic compounds and their activity, the total phenol content was determined using the Folin-Cioclateu method with slight modifications **(Singleton et al., 1999)**. The extract solution (1.0 mL, 1 mg/mL) was mixed with the Folin–Ciocalteu reagent (10 mL, previously diluted in water 1:10, v/v) and sodium carbonate (Na_2CO_3; 8.0 mL, 75 g/L). The tubes containing the solutions were vortexed for 15 s and incubated for 120 min at room temperature for color development. The absorbance was measured at 765 nm against a blank (methanol) using a UV–VIS spectrophotometer (Varian Cary 60, Agilent). All assays were performed in triplicate. Gallic acid (0.0100–0.150 mg/mL) was used for calibration and the results were expressed as mg of gallic acid equivalents per gram of dry extract (mg GAE/g).

2.6.2 Alkaloid quantification

Methods for quantification of total alkaloidal content include titrimetric methods using different types of dyes as indicators or colorimetric methods involving reagents like cobalt thiocyanates (**Deltombe et al., 1962; Nerin et al., 1985**), Reinecke salts (**Kuchta et al., 2013**) or Dragendorff reagent **(Paech and Tracey, 1955)**. Titrimetric methods are time consuming with user dependent variation, while Reinecke salts and cobalt thiocyanate salts reagents utilized in spectrophotometric methods are not suitable for routine alkaloid detection (**Sreevidya and Mehrotra, 2003)**. Dragendorff reagent (DR) is used in thin-layer chromatography for detection of alkaloids and was modified to a spectrophotometric method for routine quantification of total alkaloids in plant extracts (**Svendsen and Verpoorte, 1983; Sreevidya and Mehrotra, 2003)**. The quantification of total alkaloids is based on precipitation of alkaloids by DR followed by the formation of a yellow bismuth complex in nitric acid medium with thiourea and applicable to individual alkaloids and alkaloid-containing plant extracts.

Dragendorff Reagent (DR) was prepared by dissolving bismuth nitrate ($Bi(NO_3)_3{\cdot}5\ H_2O$; 0.80 g) in 40 mL distilled water and 10 mL glacial acetic acid. The resulting solution was mixed with potassium iodide (20.0 mL, 400 g/L). The aqueous extract was dissolved in 0.01 M HCl, while the other extracts were dissolved using a mixture of methanol with 2 % acetic acid (v/v). The extract solution (5.0 mL, 10 mg/mL) was adjusted to 40 mL using 0.01 M HCl, and 2 mL of DR was added to form an orange precipitate that was centrifuged at 3200 rpm for 15 min. The supernatant was decanted, and the residue was treated with sodium sulfide (2 mL, 10 g/L) to form a black precipitate which was centrifuged at 3200 rpm for 15 min. The resulting residue was dissolved in nitric acid (2 mL, 70 %) by warming and sonication and filled up to 10 mL with distilled water. Thiourea (5 mL, 30 g/L) was added to 1 mL of the resulting solution to form a yellow bismuth complex, of which the absorbance was measured at 465 nm against the blank (water) using a UV–VIS spectrophotometer (Varian Cary 60, Agilent). All assays were performed in triplicate. The alkaloid concentration present in the extracts was calculated from the calibration curve of bismuth nitrate (0.050 – 0.500 bismuth µmol/mL). The results were expressed as mg of boldine equivalents per gram of dry extract (mg BE/g), considering that this is a monobasic alkaloid, and therefore the complex formed with bismuth follows a 1:1 stoichiometry.

2.6.3 Triterpenoid saponin quantification

Most commonly selected spectrophotometric methods for quantification of total triterpenoid-saponins are based on the reaction of oxidized triterpenoid saponins by sulfuric acid or perchloric acid with vanillin (**Li et al., 2010; Hiai et al., 1976; Chen et al., 2007; Wu et al., 2001**). The differences in selection of reagents, standards for calibration, wavelength and different conditions to allow full color development, makes it difficult to compare the results from previous researches but provide good reference for experimental design **(Cheok et al., 2014)**. Most researchers selected 60 °C to allow full color development measured at a wavelength of 544 nm. The triterpenoid content was determined by utilizing the described parameters with slight modifications **(Oludemi et al., 2018)**.

The test solution (200µL, 1mg/mL) in a 10 mL tube was heated to evaporate the solvent and reconstituted the solid in a vanillin-glacial acetic acid solution (300 µL, 5 % w/v) and perchloric acid (1.0 mL, 70 %). The sealed samples were heated for 45 min at 60 °C and afterwards cooled in ice-water bath followed by the addition of glacial acetic acid (4.5 mL). The absorbance of the sample solutions was measured at 540 nm against a blank using a UV–VIS spectrophotometer (Varian Cary 60, Agilent). The blank was treated identically with the exception that no vanillin was used. Oleanolic acid (0.0090 – 0.4000 mg/mL in methanol) was used for calibration and the results were expressed as mg of oleanolic acid equivalents per gram of dry extract (mg OAE/g).

2.6.4 Steroidal saponin quantification

Methods used for the spectrophotometric quantification of steroidal-saponin content in plant materials are primary based on quantification of steroidal sapogenins assuming stable and reproducible results obtained with a number of standards, i.e. diosgenin, tigogenin, hecogenin, etc., without interference from sugars, sterols, fatty acid and vegetable oil and was therefore chosen as the method in this study with minor modifications **(Baccou et al., 1977; Ncube et al., 2011; Wang and McAllister, 2010).**

After the test solution (200µL, 1mg/mL) in a 10 mL tube was heated to evaporate the solvent, the precipitate was dissolved in ethyl acetate (2.0 mL) and mixed with anisaldehyde/ethyl acetate solution (1.0 mL, 0.5 %, v/v) and sulfuric acid (1.0 mL, previously diluted in ethyl acetate 1:1, v/v). The sealed samples were further incubated for 20 min at 60 °C and cooled afterwards in ice-water bath followed by the addition of demineralized water (0.5 mL). The samples were incubated for 30 min before the absorbance of the sample solutions was measured at 430 nm against a blank using a UV–VIS spectrophotometer (Varian Cary 60, Agilent). The blank was treated identically with the exception that no anisaldehyde was used. Diosgenin (0.0125 – 0.2000 mg/mL in methanol) was used for calibration and the results were expressed as mg of diosgenin equivalents per gram of dry extract (mg DE/g).

2.7 Statistical analysis

All results are expressed as means ± standard deviation (SD). Statistical comparisons were performed using Prism 7 software (GraphPad Software, La Jolla, Calif). For tiliroside and secondary metabolites content the differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1 Yield and tiliroside quantification

The study aimed at identifying most suitable extraction parameters to obtain extracts with high tiliroside and extract yield . The results obtained are summarized in **Table 2**.

Table 2: Summary of extract yield and tiliroside content of Waltheria Indica leave extracts obtained varying extraction temperatures and solvents.

Sample	W90	M90	E30	E90	E150	EA90
Yield extract [mg/g plant]	227.2	218.4	16.0	110.7	171.9	29.0
Tiliroside [mg/g extract]	2.47 ± 0.27[a]	18.09 ± 0.83[b]	0.56 ± 0.01[c]	24.60 ± 0.45[d]	19.69 ± 0.15[e]	3.43 ± 0.08[a]
Tiliroside [mg/g plant]	0.56	3.95	0.0089	2.72	3.39	0.10

Values are means ± standard deviations of triplicate measurements. W90, 90 °C water; M90, 90 °C methanol; E30-E90-E150, 30-90-150 °C ethanol; EA90, 90 °C ethyl acetate; n.d., not detectable. Values in a row with different letters are significantly different ($p < 0.05$; one-way ANOVA).

After complete drying of the extracts, the extract yield was calculated according to **equation 1**. The highest yield at 90 °C extraction temperature was achieved with water (227.2 mg/g) and methanol (218.4 mg/g) as solvent. With ethanol (110.7 mg/g) the yield is two-fold lower, while the lowest amount was extracted using ethyl acetate (29.0 mg/g). Ethanol extracts obtained at different temperatures show an increase in yield with 16.0 mg/g, 110.7 mg/g and 171.9 mg/g as the temperature was increased from 30 °C to 90 °C and 150 °C respectively.

Quantification of tiliroside concentration revealed that at 90 °C extraction temperature the ethanol extract with 24.60 mg/g and the methanol extract with 18.09 mg/g contained the highest amount of tiliroside. In water and ethyl acetate extracts, tiliroside is present in smaller quantities with 2.47 mg/g and 3.43 mg/g respectively. Ethanol extracts obtained at different temperature conditions contain 0.56 mg/g tiliroside at 30 °C, 24.60 mg/g at 90 °C and 19.69 mg/g at 150 °C.

To determine the total amount of tiliroside extractable from the plant, tiliroside yield in mg/g plant was calculated according to **equation 2**. At 90 °C the highest amount of tiliroside extracted per gram plant material was achieved with methanol as solvent (3.95 mg), followed by ethanol (2.72 mg), water (0.56 mg) and ethyl acetate (0.10 mg). Rising the extraction temperature using ethanol as solvent resulted in increased extracted amounts of tiliroside with 0.0089 mg, 2.72 mg and 3.39 mg at 30 °C, 90 °C and 150 °C respectively.

3.2 COX-2 inhibitory activity

The results of relative COX-2 inhibition of pure tiliroside are shown in **Figure 2**. Starting at a concentration of 1.5 µM tiliroside COX-2 inhibition increased in a dose dependent manner up to 51.2 %

for 150µM tiliroside, with the lowest concentration showing significant activity vs. blank being 15 µM.

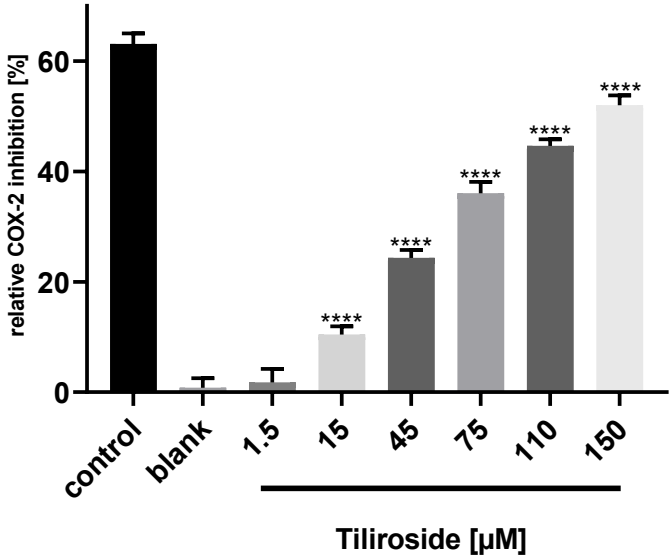


Figure 2: COX-2 inhibition (%) of tiliroside, blank (DMSO) and control (0.5 µM celecoxib). Data points represent the mean value ± standard deviations of six samples. Statistically different expressions were calculated using one-way ANOVA: (****): $p < 0.0001$ vs. blank.

The extracts were sorted according to their increasing tiliroside content, with E30 containing the lowest and E90 the highest tiliroside content and plotted against their respective COX-2 inhibition activity in **Figure 3**. The results include further a threshold line at 30% COX-2 inhibition, which is assumed as a minimal *in-vitro* inhibitory efficacy to translate into a measurable effect *in-vivo* (data not shown). Extracts above this level (EA90, E30 and E150) are assumed to have inhibitory potential in cell culture and *in-vivo*.

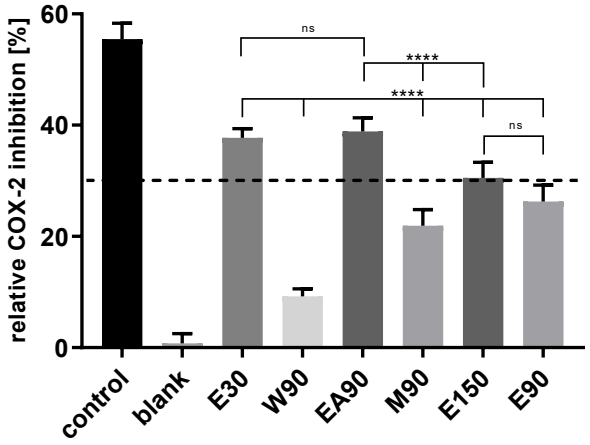


Figure 3: COX-2 inhibition (%) of Waltheria Indica extract solution (20 µg/mL), blank (DMSO), control (0.5 µM celecoxib) and threshold (dashed line) at 30% COX-2 inhibition. Data points represent the mean value ± standard deviations of six samples. Statistically different expressions were calculated using one-way ANOVA: (****): $p < 0.0001$, (ns): not significant.

The extract E90 with the highest tiliroside content (24.6 mg/g extract) corresponded to a concentration of 0.8 µM pure tiliroside in the assay, which is below the threshold concentration to observe a COX-2 inhibition. The extract M90 showed at a concentration of 20 µg/mL a similar COX-2 inhibition to tiliroside at 75 µM (45 µg/mL) which means that it may comprise compounds which are by a factor of 2.3 more potent than tiliroside. The extracts EA90 and E30 at 20 µg/ml, show a similar COX-2 inhibition to tiliroside at 110 µM (65 µg/mL) indicating that these extracts contain compounds which are by a factor 3.3 more potent than tiliroside.
To further substantiate this assumption and to characterize the COX-2 inhibiting compounds, further investigations regarding the secondary metabolite profiles of the extracts were carried out in the following part of the study.

3.3 Quantitative determination of secondary metabolites

3.3.1 Phenol quantification

Phenol concentration in the respective extracts is summarized with the other secondary metabolites in **Table 3**. At 90 °C extraction temperature the ethanol extract with 148.7 mg/g, methanol with 142.6 mg/g and water with 128.3 mg/g contained the highest amount of phenolic compounds, while phenols in ethyl acetate extract are present in smaller quantities with 45.6 mg/g. Ethanol extracts obtained under varying temperature conditions demonstrated the highest phenol content at 30 °C with 188.0 mg/g, while at 90 °C and 150 °C the amount was 148.7 mg/g and 148.8 mg/g respectively.

3.3.2 Alkaloid quantification

No alkaloid content at 90 °C extraction temperature was detected in water and ethyl acetate extracts. However, the methanol extract contained 1.3 mg/g alkaloid, while ethanol extracts showed an extraction temperature dependence and the concentration increased from 5.2 mg/g at 30 °C to 6.4 mg/g at 90 °C and 10.0 mg/g at 150 °C **(Table 3)**.

3.3.3 Triterpenoid saponin quantification

At 90 °C extraction temperature the highest amount of triterpenoid saponins (195.0 mg/g) was found in the ethyl acetate extract, followed by ethanol (143.6 mg/g), methanol (106.0 mg/g) and water (47.3 mg/g). Ethanol extractions at different temperatures demonstrated that the highest amount of triterpenoid saponins (214.8 mg/g) was extracted at 30 °C, followed by 90 °C and 150 °C extracts with 143.6.5 mg/g and 120.1 mg/g respectively **(Table 3)**.

3.3.4 Steroidal saponin quantification

At 90 °C extraction temperature the highest amount of steroidal saponin (113.6 mg/g) was found in ethyl acetate extract, followed by ethanol (57.7 mg/g), methanol (53.0 mg/g) and water (7.4 mg/g). Ethanol extractions at different temperatures demonstrated that the highest amount of steroidal saponin (85.1 mg/g) was extracted at 30 °C, followed by extractions at 90 °C and 150 °C with 57.7 mg/g and 59.2 mg/g respectively **(Table 3)**.

Table 3: Secondary metabolite content and yield of Waltheria Indica leave extracts obtained by varying extraction temperatures and solvents. (GAE=Gallic acid equivalent; BE=Boldine equivalent; OEA=Oleanolic acid equivalent; DE=Diosgenin equivalent).

Sample	W90	M90	E30	E90	E150	EA90
Total-Phenols [mg GAE/g]	128.3 ± 4.8^{a}	142.6 ± 2.2^{b}	188.0 ± 3.0^{c}	148.7 ± 3.2^{b}	148.8 ± 2.2^{b}	45.6 ± 1.2^{d}
Total-Alkaloids [mg BE/g]	n.d.	1.3 ± 0.1^{a}	5.2 ± 0.2^{b}	6.4 ± 0.1^{c}	10.0 ± 0.2^{d}	n.d.
Triterpenoid-saponins [mg OAE/g]	47.3 ± 2.1^{a}	106.0 ± 3.9^{b}	214.8 ± 2.3^{c}	143.6 ± 2.5^{d}	120.1 ± 3.6^{e}	195.0 ± 3.6^{f}
Steroidal-saponins [mg DE/g]	7.4 ± 0.7^{a}	53.0 ± 0.8^{b}	85.1 ± 1.3^{c}	57.7 ± 1.5^{d}	59.2 ± 1.4^{d}	113.6 ± 2.7^{e}

Values are means ± standard deviations of triplicate measurements. W90, 90 °C water; M90, 90 °C methanol; E30-E90-E150, 30-90-150 °C ethanol; EA90, 90 °C ethyl acetate; n.d., not detectable. Values in a row with different letters are significantly different ($p < 0.05$; one-way ANOVA).

Direct positive correlation was observed between COX-2 inhibition and triterpenoid- and steroidal-saponin concentration. Extracts with higher triterpenoid- and steroidal-saponins concentrations result in increased COX-2 inhibition (**Figure 4**).

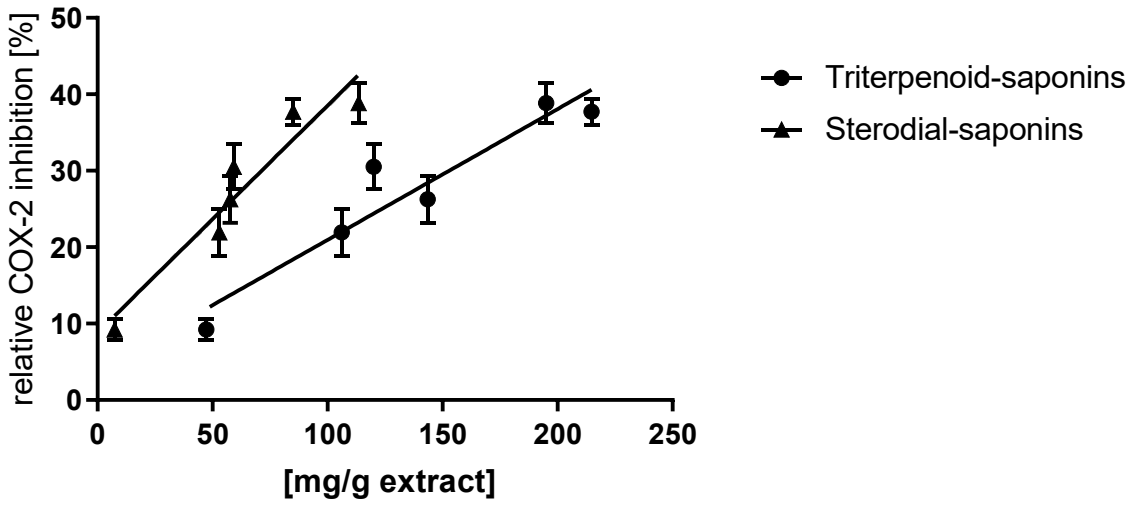
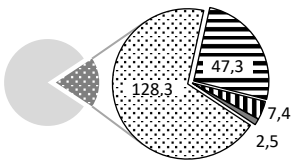
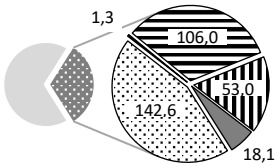
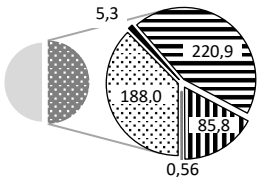
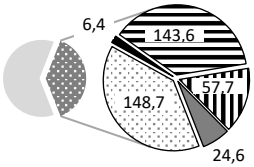
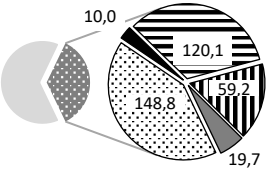
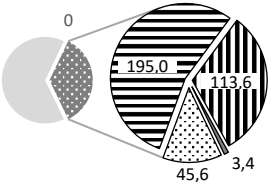


Figure 4: Graphs represent the mean value of COX-2 inhibition (%) at 20µg/mL extract solution versus triterpenoid-saponin and steroidal-saponin content. The correlation coefficient values for triterpenoid-saponin content (R^2 = 0.8592, $p<0.0001$) and steroidal-saponin content (R^2 = 0.8691, $p<0.0001$) were observed at a 95 % confidence level.

The composition of the extracts with their corresponding COX-2 inhibition as well as the amount of uncharacterized compounds, which may include *e.g.* carbohydrates (like cellulose, starch), lipids, chlorophyll, lactones, salts, polypeptides, RNA and DNA are summarized in **Table 4**.

Table 4: Summary of relative polarity of used solvents, Waltheria Indica leaf extract yield at varying extraction temperatures and solvents; secondary metabolite profile as overview of relative composition and absolute values (mg/g extract), characterized and uncharacterized metabolite quantities; COX-2 inhibition (%) of Waltheria Indica extract solution (20 µg/mL), extract mass equivalent per millilitre to inhibit 30 % COX-2 activity ($ME_{30\%COX\text{-}2}$) assuming a linear relationship and plant material required to obtain 1 g extract. Tiliroside is also detected by the phenol-assay and listed separately for ease of comparison. W90, 90 °C water; M90, 90 °C methanol; EA90, 90 °C ethyl acetate; E30-E90-E150, 30-90-150 °C ethanol.

Sample	W90	M90	E30	E90	E120	EA90
Solvent polarity	1	0.76	0.65	0.65	0.65	0.23
Yield extract [mg/g plant]	227	218	16	111	172	29
Metabolites overview [mg/g extract]						
Metabolites characterized [mg/g extract]	185.5	321.0	500.6	381.0	357.8	357.6
Metabolites uncharacterized [mg/g extract]	814.5	679.0	499.4	619.0	642.2	642.4
Inhibition COX-2 [%]	9.2	21.9	37.7	26.3	30.5	38.9
$ME_{30\%COX\text{-}2}$ [µg extract/mL]	65.2	27.4	15.9	22.8	19.7	15.4
Plant required [g/g extract]	4.4	4.6	62.4	9.0	5.8	34.4

uncharacterized characterized Phenols Alkaloids Triterpenoid-saponins Steroidal-saponins Tiliroside

4. DISCUSSION

Tests carried out on *Waltheria Indica* leaves revealed the influence of the solvents used and temperatures on the extract and tiliroside yield. The presence of tiliroside in plant extracts is often associated with anti-inflammatory properties, while investigations on the influence of extraction parameters on the tiliroside concentration from *Waltheria Indica* leaves and its correlation to the contribution of anti-inflammatory properties have not been reported yet (**Grochowski et al., 2018)**.

At 90 °C extraction temperature the highest extract yield is obtained with more polar solvents (water and methanol) compared to extracts obtained with solvents of lower polarity (ethanol and ethyl acetate). The investigations of ethanolic extracts with varying extraction temperatures demonstrated an increase of yield at higher temperatures (**Table 2**). The analysis of the tiliroside content revealed that extracts obtained at 90 °C contained the highest amount of tiliroside when using the medium-polar solvent ethanol, followed by methanol and significantly lower quantity when extracted with polar (water) or non-polar (ethyl acetate) solvent.

The extraction with ethanol revealed that the total yield of extracted tiliroside per gram of plant material increased at elevated temperatures. In contrast, an extract obtained at 90 °C contained the highest tiliroside content, whereas the amount is significantly lower at 150 °C and 30 °C respectively (**Table 2**). At room temperature it is assumed that the solvent does not sufficiently penetrate the cell wall and the lipid bilayer cell membrane to dissolve tiliroside. At higher temperature the solvent penetrates more efficiently, and additional substances are extracted leading to a dilution of tiliroside in the total extract.

Pure tiliroside inhibit in a concentration-dependent manner the COX-2 activity, with the lowest concentration showing significant activity being 15 µM (**Figure 2**). However, the correlation between tiliroside content and COX-2 inhibition could not be confirmed for the extracts. Extracts with the highest tiliroside content (E90, E150) did not exhibit the highest COX-2 inhibition, in contrast highest inhibitory activity was found in extracts with low tiliroside content (EA90, E30) (**Figure 3**). One reason might be that even the extract with the highest tiliroside content (E90) corresponded to 0.8 µM pure tiliroside, which was below a measurable activity (**Figure 2**). Comparison of the extracts M90, EA90 and E30 with pure tiliroside at comparable COX-2 inhibition levels indicates that M90 contain metabolites with factor 2.2 and EA90, E30 with factor 3.3 higher COX-2 inhibition activity than tiliroside (**Table 4**). These results demonstrate that the COX-2 inhibition of the extracts is not caused primarily by the tiliroside content, but by other potent metabolites.

The extract quantity and composition of secondary metabolites depend on the solvent used for extraction. With respect to the solvent polarity, different secondary metabolites are preferentially extracted as summarized in **Table 5** (**Eloff, 1998; Cowan, 1999; Rafińska et al., 2019; Wakeel et al., 2019**).

Table 5: Overview of secondary metabolites extracted with solvents as reported by **Cowan (1999)** and modified regarding the replacement of chloroform with ethyl acetate due to similarity in relative polarity. [1]part of the uncharacterized metabolite concentration; [2]part of the total-phenol concentration; [3]quantification of triterpenoid-saponin types; [4]quantified separately as triterpenoid- and steroidal saponins; [5]quantified as total-alkaloid content.

Solvent	Water	Methanol	Ethanol	Ethyl acetate
Relative polarity	1	0.76	0.65	0.23
Secondary metabolites	Starches[1]	Lactones[1]	Tannins[2]	Terpenoids[3]
	Phenols[2]	Tannins[2]	Polyphenols[2]	Saponins[4]
	Terpenoids[3]	Polyphenols[2]	Terpenoids[3]	Flavonoids[2]
	Polypeptides[2]	Terpenoids[3]	Alkaloids[5]	
	Saponins[4]	Alkaloids[5]	Saponins[4]	
		Saponins[4]		

The composition of the secondary metabolites of the extracts revealed that, except for ethyl acetate as solvent, the phenol concentrations varies minor using different solvents or temperatures. The two extracts with the highest COX-2 inhibition activity (EA90, E30) are with the lowest (EA90) and the highest (E30) phenol content respectively, demonstrating that there is no correlation between phenol content and COX-2 inhibition (**Table 4**)

The analyses of the alkaloid content in the extracts revealed low or undetectable concentrations compared to the other secondary metabolites studied. The highest alkaloid concentrations were present in the ethanolic extracts and increased at elevated extraction temperatures, while no alkaloid could be detected in ethyl acetate and water extracts. Comparison of alkaloid content and COX-2 inhibition activity of the extracts revealed no correlation. The extract with the highest alkaloid concentration (E150) performs significantly weaker than the extract with the best inhibition (EA90) where the alkaloid concentration is below the detection limit (**Table 4**).

The investigations of triterpenoid- and steroidal-saponin content in the individual extracts revealed a direct correlation to the solvent polarity: the content of saponins increases with reduced polarity of the solvent (ethyl acetate > ethanol > methanol > water). Being composed of an aglycon (water-insoluble part) attached to oligosaccharide units (water-soluble part), triterpenoid and steroid saponins are extracted more efficiently depending on the aglycon structure and number of sugar moieties attached (**Hostettmann and Marston, 1995; Cheok et al., 2014; Ms et al., 2018**). The results demonstrate a more efficient extraction with non-polar solvents indicating that saponins in *Waltheria Indica* leaves are mainly present as aglycones or contain a minor amount of sugar moieties. The extraction with ethanol at different temperatures show that a lower temperature lead to a higher content of triterpenoid- and steroidal-saponins (**Table 3**). This might be explained by the fact that saponins dissolve well in ethanol, but as the temperature of extraction increases, further substances are extracted from the plant material and the relative proportion of saponins in the final extract decreases.

Furthermore, a surprisingly direct positive correlation was observed between COX-2 inhibition and triterpenoid- and steroidal-saponin concentration. Extracts with higher triterpenoid- and steroidal-saponins concentrations result in increased COX-2 inhibition. Based on the slope of the linear

regression, the results indicate that steroidal-saponins of *Waltheria Indica* extracts are more potent COX-2 inhibitors compared to triterpenoid-saponins (**Figure 4**).

These findings are of importance as the anti-inflammatory properties of *Waltheria Indica* extracts are commonly linked to flavonoids such as tiliroside or quercetin (**Zongo et al., 2013; Grochowski et al., 2018; Laczko et al., 2019**). Although it has been proven that these flavonoids possess anti-inflammatory properties, the required concentration in the extract need to be increased to exhibit a significant detectable anti-inflammatory activity (**Figure 2**). Besides flavonoids such as tiliroside, also triterpenoid- and steroidal-saponins are reported to exhibit COX-2 inhibition (**Jachak, 2006; Desai et al., 2018; Han et al., 2013; Wu et al., 2017; Wand and Meng, 2015**). Several triterpenoids including (3β)-3-Hydroxy-lup-20(29)-en-28-oic acid (betulinic acid), β-acetoxy-27-trans-caffeoyloxyolean-12-en-28-oicacid methyl ester and β-acetoxy-27-cis-caffeoyloxyolean-12-en-28-oic acid methyl ester have been recently isolated from the aerial parts of *Waltheria Indica* (**Cretton et al., 2015; Monteillier et al., 2017; Caridade et al., 2018**) but without specifying their concentrations in these extracts.

This study using *Waltheria Indica* revealed that its leave extracts, as well as its major compound tiliroside, are efficient in regulating inflammatory immune responses by inhibiting the enzymatic activity of COX-2 known to be involved in skin inflammatory conditions and diseases such as arthritis, allergic contact and atopic dermatitis development **(Crofford, 1999**; **Greaves and Camp, 1988; Robb et al., 2018; Seo et al., 2003)**. Furthermore, the results demonstrate that substances from the group of triterpenoid- and steroidal-saponins are directly involved in the COX-2 inhibition activity of the extracts.

To establish optimal extraction conditions for Waltheria Indica resulting in an extract with highest COX-2 inhibition one should identify the substances responsible for the direct or synergistic efficacy. Further investigations are required to clarify whether this anti-inflammatory activity originates from individual saponins at a defined concentration and to which extent these saponins contained in the leaves of *Waltheria Indica* might play a role in other anti-inflammatory pathways different from the arachidonic pathway.

5. CONCLUSION

The results obtained in this study show that *Waltheria Indica* extracts inhibit the inflammatory key mediator COX-2. The activity is related to the extraction parameters governing the composition of the extract. Tiliroside demonstrates COX-2 inhibitory properties, with triterpenoid- and steroidal-saponins contributing in a concentration-dependent matter. Therefore, the optimal extraction parameters for extracts with highest COX-2 inhibition cannot be determined on the basis of the tiliroside content in the extract solely. Extracts with high inhibitory activity described in this research will serve as a starting point for structural elucidation and COX-2 inhibition determination of the individual saponins in future studies.

Authors contribution

Michael Termer designed and did the experiments as well as wrote the manuscript; Joerg von Hagen contributed significantly to analysis and manuscript preparation; Christophe Carola, Andrew Salazar, Cornelia M. Keck and Juergen Hemberger assisted in the revision of the manuscript and provided valuable ideas. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they do not have any conflict of interest.

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SUPPLEMENTAL MATERIAL AND METHODS

Identification of tiliroside in Waltheria Indica extract

Identification of tiliroside in *Waltheria Indica* extracts were carried out on Vanquish HPLC system coupled with Thermo Fusion™ Orbitrap Tribrid™ mass spectrometer (Thermo Scientific™) with H-ESI interface and positive ionization. Separation was accomplished using C-18 column (Thermo Acclaim™ 120, 100 x 4.6 mm, 5 µm) and gradient elution with solvent A: water/acetonitrile/trifluoroacetic acid 95/5/0.125 (v/v/v) and B: water/acetonitrile/trifluoroacetic acid 5/95/0.125 (v/v/v). Gradient started at 7% (v/v) B, ramping up to 28 % over 15 min, ramping up to 56 % over 5 min, further to 95 % over 5 min, held for 3 min and ramping down to 7 % over 8 min. UV detection was performed at 315 nm, 25 °C column temperature, 1.5 ml/min flow speed and 10 µL injection volume.

UV-HPLC profile of *Waltheria Indica* extract obtained with ethanol at 90 °C show an intense UV peak at R_t = 14.47 min (**Figure 1**), which corresponds to tiliroside standard (data not shown).

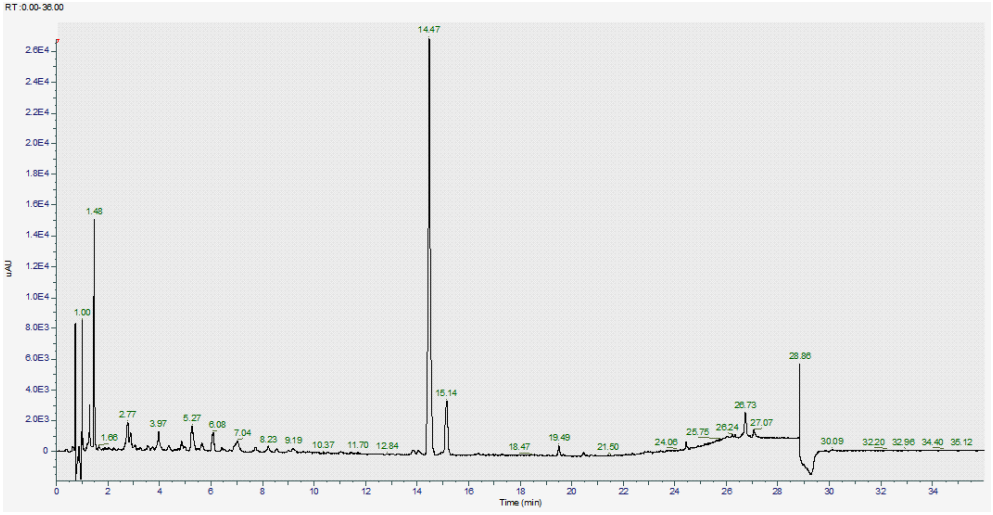

Figure 1: UV-HPLC profile at 315 nm of *Waltheria Indica* extract obtained with ethanol at 90 °C.

MS data in **Figure 2** of the peak at R_t = 14.47 show a single charge state MH+ 595.1432 g/mol and MNa+617.1249 g/mol, which corresponds to that of tiliroside.

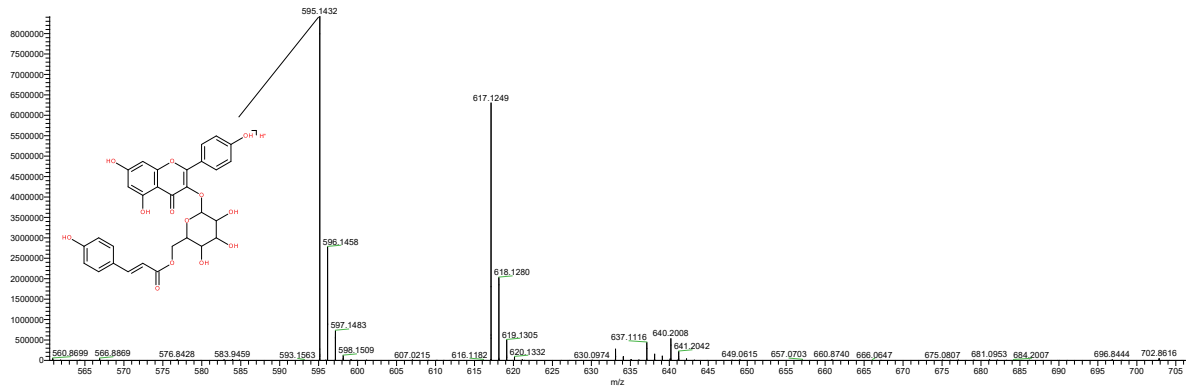

Figure 2: MS data of the peak at R_t = 14.47.

Fragments of 594.1432 g/mol signal correspond to tiliroside fragments (**Figure 3**).

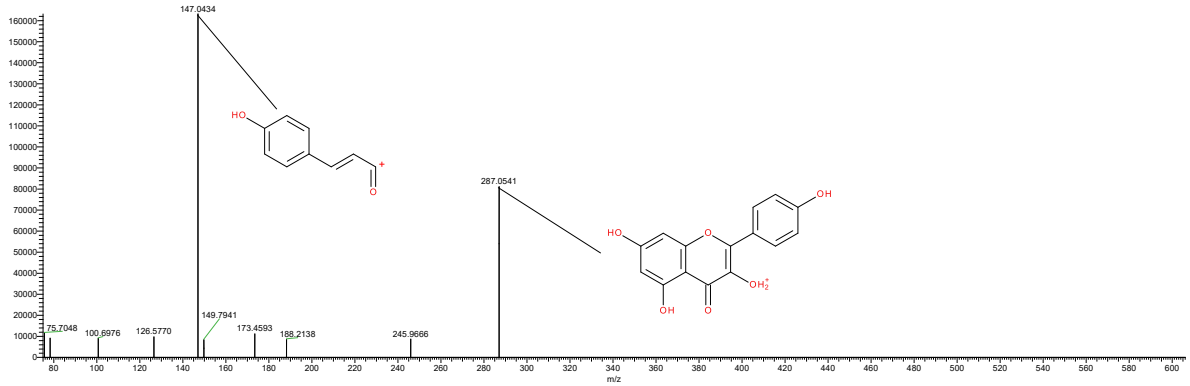

Figure 3: Fragments of 595.1432 g/mol signal with their corresponding structures.

5.2

Activity-Guided Characterization of COX-2 Inhibitory Compounds in *Waltheria Indica L.* Extracts

Activity-Guided Characterization of COX-2 Inhibitory Compounds in Waltheria Indica L. Extracts

Michael Termer[1], Christophe Carola[2], Andrew Salazar[2], Cornelia M. Keck[1], Juergen Hemberger[3], Joerg von Hagen[2,3]

[1] Department of Pharmaceutics and Biopharmaceutics, Philipps-University of Marburg, Robert-Koch-Str. 4, 35032 Marburg, Germany; cornelia.keck@pharmazie.uni-marburg.de (C.M.K.); michael.termer@pharmazie.uni-marburg.de (M.T.)

[2] Merck KGaA, Frankfurterstr. 250, 64293 Darmstadt, Germany; christophe.carola@merckgroup.com (C.C.); andrew.salazar@merckgroup.com (A.S.); joerg.von.hagen@merckgroup.com (J.v.H.)

[3] Department of Life Science Engineering, Institute for Biochemical Engineering & Analytics, University of Applied Sciences, Wiesenstr. 14, 35390 Giessen, Germany; juergen.hemberger@lse.thm.de

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Abstract

Inflammation is the body's response to infection or tissue injury in order to restore and maintain homeostasis. Prostaglandin E2 (PGE-2) derived from arachidonic acid (AA), via up-regulation of cyclooxygenase-2 (COX-2), is a key mediator of inflammation and can also be induced by several other factors including stress, chromosomal aberration, or environmental factors. Targeting prostaglandin production by inhibiting COX-2 is hence relevant for the successful resolution of inflammation. Waltheria indica L. is a traditional medicinal plant whose extracts have demonstrated COX-2 inhibitory properties. However, the compounds responsible for the activity remained unknown. For the preparation of extracts with effective anti-inflammatory properties, characterization of these substances is vital. In this work, we aimed to address this issue by characterizing the substances responsible for the COX-2 inhibitory activity in the extracts and generating pre-diction models to quantify the COX-2 inhibitory activity without biological testing. For this purpose, an extract was separated into fractions by means of centrifugal partition chromatography (CPC). The inhibitory potential of the fractions and extracts against the COX-2 enzyme was determined using a fluorometric COX-2 inhibition assay. The characterizations of compounds in the fractions with the highest COX-2 inhibitory activity were conducted by high resolution mass spectrometry (HPLC-MS/MS). It was found that these fractions contain alpha-linolenic acid, linoleic acid and oleic acid, identified and reported for the first time in Waltheria indica leaf extracts. After analyzing their contents in different Waltheria indica extracts, it could be demonstrated that these fatty acids are responsible for up to 41% of the COX-2 inhibition observed with Waltheria indica extract. Additional quantification of secondary metabolites in the extract fractions revealed that substances from the group of steroidal saponins and triterpenoid saponins also contribute to the COX-2 inhibitory activity. Based on the content of compounds contributing to COX-2 inhibition, two mathematical models were successfully developed, both of which had a root mean square error (RMSE) = 1.6% COX-2 inhibitory activity, demonstrating a high correspondence between predicted versus observed values. The results

of the predictive models further suggested that the compounds contribute to COX-2 inhibition in the order linoleic acid > alpha linolenic acid > steroidal saponins > triterpenoid saponins. The characterization of substances contributing to COX-2 inhibition in this study enables a more targeted development of extraction processes to obtain Waltheria indica extracts with superior anti-inflammatory properties.

1. Introduction

Inflammation is a defensive response of the body to various stimuli and is a cyclic, self-stimulating process designed to combat infection or tissue injury [1,2]. The inflammatory process is accompanied by the release of pro-inflammatory cytokines and prostaglandins, and the formation of reactive oxygen species (ROS) [3]. Important modulators of inflammation are nuclear factor kappa B (NF-kB), lipoxygenase (LOX) and cyclooxygenase (COX), with NF-kB activating the expression of LOX or COX [4,5]. Both enzymes are part of the arachidonic acid (AA) metabolism, one of the main cellular processes for mediating inflammation, where they exhibit a catalytic effect. The COX-1 and COX-2 enzymes are isozymes, and in normal human skin, COX-1 is present through the epidermis, whereas COX-2 localizes mainly in suprabasal keratinocytes [6–8]. While COX-1 is in-volved in homoeostatic processes and expressed constitutively in most tissues, the pro-inflammatory COX-2 is an inducible isoform and is mainly produced in inflamed tissues. To maintain and restore homeostasis of the skin, different cellular components regulate local immune responses thorough crosstalk [2,9]. The initiation and the maintenance of the inflammation is carried out by pro-inflammatory mediators. Once the instigating factor is removed, activity is balanced out by the anti-inflammatory mediators re-sponsible for limiting the inflammation [10,11]. Several factors including stress, chromosomal aberration or environmental factors disturb this balance and lead to excessive pro-duction of prostaglandin E2 (PGE2) derived from arachidonic acid, via the up-regulation of COX-2, consequently leading to inflammatory-mediated diseases [12–16].

Increased PGE2 levels lead to increased production of proinflammatory cytokines such as interleukin-6 (IL-6), with neutrophils and macrophage immune cells responding. As a result of the IL-6 concentration, the amplification of inflammation-related signals or transformation from an acute to a chronic inflammatory state occurs [17,18]. Targeting prostaglandin production by inhibiting COX-2, the rate-limiting enzyme, is one of the options to successfully treat inflammatory skin diseases [8,19].

Waltheria indica L., belonging to the Malvaceae family, is a traditional medicinal plant with anti-inflammatory properties used by indigenous populations in different regions of the world for the treatment of various pathological conditions [20,21]. The biologically active compounds proved to be present in all parts of Waltheria indica with reports supporting the use of roots, stems or leaves for treatment against swelling, cough, toothache, sore throat, rheumatism or complicated ailments such as asthma and inflammatory skin dis-eases [21–25]. Studies on the properties of Waltheria Indica showed that its extracts have multiple anti-inflammatory activities, often without attributing the effect to a single molecule. Extracts that exhibit biological activity include various chemical groups such as alkaloids, flavonoids, sterols, terpenes, anthraquinones or carbohydrates [21,26,27]. Hydroalcoholic extracts of the whole plant of Waltheria indica, for example, strongly inhibited edema at the second phase of

carrageenan inflammation in rats [28]. Extracts from leaves generated with hydrophobic solvents such as petrol ether or methanol, but also extracts from leafy stems generated with hydrophilic solvents such as water, showed dose-related inhibition of acute and chronic inflammation in carrageenan-induced edema. The effect is presumed to involve the inhibition of histamine, serotonin, bradykinin, prostaglandin and cyclooxygenase (COX) products [29–31].

Studies that aimed at uncovering the anti-inflammatory molecules in Waltheria identified three flavonoids ((-)-epicatechin, quercetin and tiliroside) and two alkaloids (waltherione A and C) as potential active molecules [21,32,33]. The alkaloids waltherione A and C, obtained from the decoction of roots and aerial parts, were shown to inhibit nuclear factor (NF-κB) [34]. Quercetin and tiliroside showed a dose-dependent inhibition of the production of inflammatory mediators, including nitric oxide (NO), tumor necrosis factor (TNF-alpha), interleukin (IL)-12 and COX-2 [32,35,36]. Furthermore, it was suggested that the reason for the observed inhibition of lipoxidase-5 (5-LOX) and phospholipase A2 (PLA2) by the hydroalcoholic Waltheria extracts is due to (-)-epicatechin, which, in other studies, demonstrated COX-2 inhibitory properties [21,33,37,38]. It is important to mention that although these molecules exhibited efficacy in the performed assays, it was not evident from these studies whether their content in the Waltheria extracts is sufficient or different substances contribute to or are responsible for the observed activity. In addition to the substances isolated from Waltheria indica, other classes of compounds have been explored in various plants that exhibit significant COX-2 inhibitory activity, with flavonoids, alkaloids, terpenoids, saponins and fatty acids representing the most prominent ones that are presumably present but not yet identified in Waltheria indica [39].

Given the complexity of the plant extracts, the substances contributing to the anti-inflammatory effect of the extracts are regularly merely assumed and, in most cases, unknown or assigned to molecules whose content in the extract is insufficient or not known at all. Consequently, in our previous study we attempted to clarify to what extent a known anti-inflammatory molecule present in Waltheria indica, namely tiliroside, contributes to the COX-2 inhibition activity in the extracts. We demonstrated that tiliroside inhibits COX-2 activity in a concentration-dependent manner. However, the correlation between tiliroside content and COX-2 inhibition could not be confirmed for the extracts. Ex-tracts with the highest tiliroside content did not exhibit the highest COX-2 inhibition; in contrast, the highest inhibitory activity was found in extracts with low tiliroside content. This led to the conclusion that in these extracts, other, more potent substances must be re-sponsible for the activity [40].

A study identifying the substances responsible for the COX-2 inhibitory activity of Waltheria indica leaf extracts is yet not described. Therefore, the aim of this study was to identify and quantify the molecules responsible for the COX-2 inhibitory activity of the ex-tracts using an activity-guided approach through extract fractionation by centrifugal partition chromatography (CPC). Ideally, it should be verified whether and to what extent the COX-2 inhibitory effect correlates with the content of these substances in the extracts. In addition, the different CPC fractions produced were analyzed for their secondary metabolite composition in terms of phenols, and triterpenoid and steroidal saponin content to ideally allow the generation of a prediction model to quantify the COX-2 inhibitory activity without biological testing.

The activity-guided approach led to the identification of three fatty acids, which were identified and reported for the first time in Waltheria indica leaf extracts. The investigations provide evidence that these fatty acids are responsible for up to 41% of the COX-2 inhibition observed in the respective Waltheria indica extract. Additional phytochemical analyses of the extract fractions revealed that substances from the group of steroidal saponins and triterpenoid saponins contribute to the COX-2 inhibitory properties of the extracts. The identification of substances contributing to COX-2 inhibition enabled the successful development of mathematical models to predict the COX-2 inhibition of Waltheria indica extracts, allowing the preparation of extracts with enhanced anti-inflammatory properties in future studies.

2. Results and Discussion

2.1. Centrifugal Partition Chromatography Fractionation

With the goal of obtaining fractions composed of different compounds of the Waltheria indica leaf extract, the separation was carried out using CPC coupled with a diode array detector (DAD). For this purpose, Waltheria leaves were extracted utilizing accelerated solvent extraction (ASE) at 70 °C with ethanol. To determine the appropriate solvent com-position for the CPC, a systematic approach was adapted as described in the literature, using the Arizona liquid system (AZ) with the solvent composition N (AZ-N) as the starting point [41]. The method was performed in two steps, where step 1 was the elution step with a column volume of mobile phase and a run-time of 50 min. Step 2 was the extrusion step, replacing the entering mobile phase with the stationary phase. Outcomes with sol-vent compositions that had either formed an unstable system or presented no added value according to the AZ system were not listed. The yields of the individual dried fractions from separate CPC runs and the recovery rate of the injected extract amount are summarized in **Table 1**.

Table 1. Yields (%, w/w) of the individual fractions (F1–F8) and the recovery rate from CPC runs with Arizona liquid system and the medium polar solvent composition N (AZ-N), non-polar solvent composition T (AZ-T) and polar solvent composition L (AZ-L); n.a., not available. Data points represent the mean value ± standard deviations of three samples.

CPC Fraction	F1	F2	F3	F4	F5	F6	F7	F8	Recovery (%)
AZ-N yield (%)	0	55.3 ± 2.4	2.8 ± 0.1	1.5 ± 0.1	39.9 ± 2.0	0	n.a.	n.a.	99.3 ± 4.2
AZ- T yield (%)	0	49.6 ± 2.4	8.7 ± 1.2	0.8 ± 0.2	13.2 ± 1.4	27.5 ± 1.8	0	n.a.	99.7 ± 3.8
AZ- L yield (%)	0	31.5 ± 1.2	25.0 ± 1.6	1.9 ± 0.2	0	2.1 ± 0.2	38.8 ± 1.4	0	99.2 ± 3.6

From the obtained yields (**Table 1**), it was evident that with AZ-N the extract was separated into two main fractions: N-F2 (55.3% yield) containing the more polar com-pounds and N-F5 (39.9% yield) containing the more nonpolar compounds. The observed signals at N-F1 and N-F4 were solvent turbulences (**Figure 1A**). For N-F1, this was due to sample injection, and for N-F4, this was due to the transition from the elution to the extru-sion step. These effects were also observed for the AZ-T and AZ-L runs at the corresponding time periods (**Figure 1B,C**).

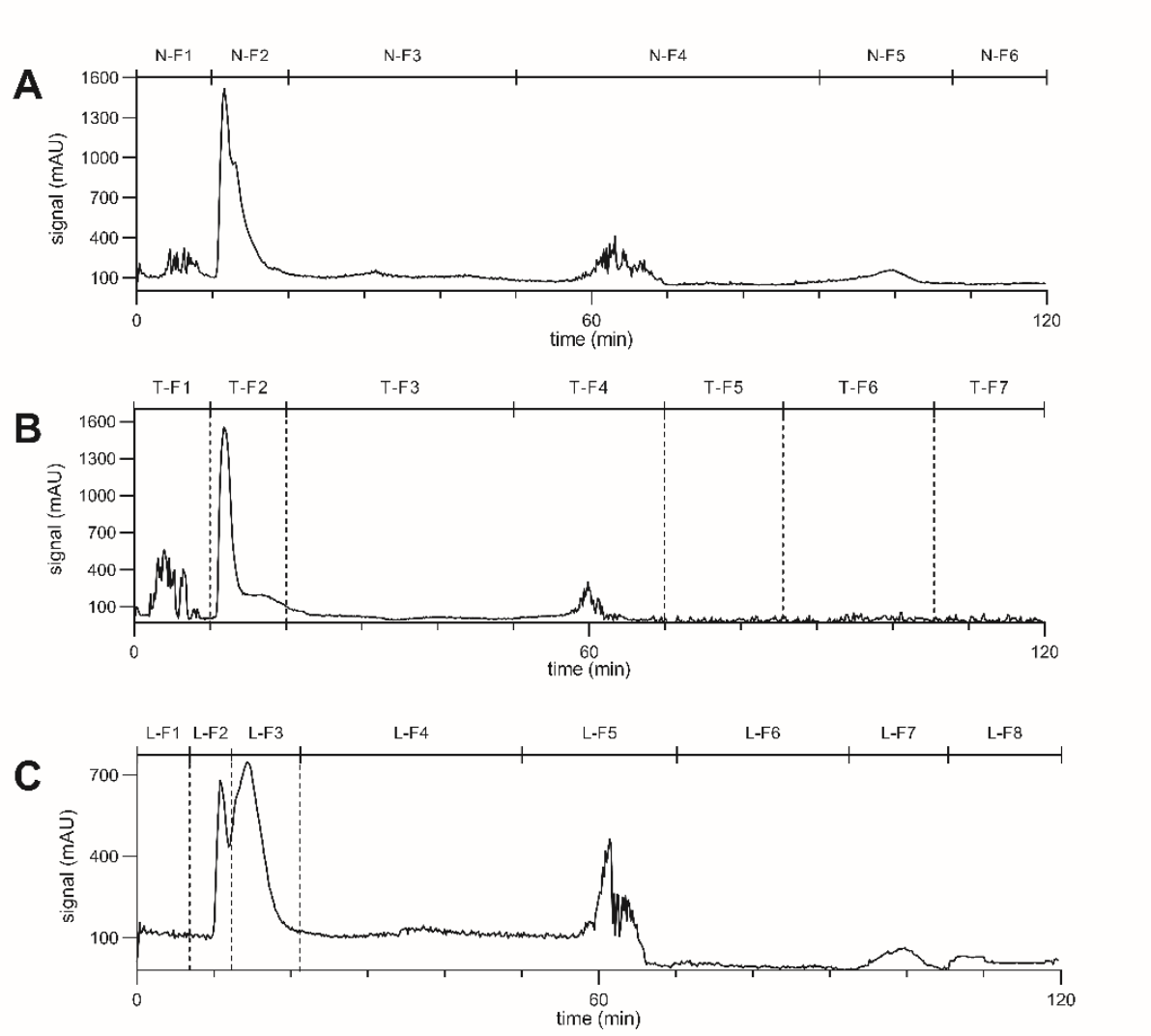

Figure 1. Chromatograms at 254 nm from CPC fractionations with Waltheria indica leaf extract and Arizona liquid system with (A) solvent composition N, (B) solvent composition T and (C) solvent composition L. The elution step lasted for 50 min initially, followed by an extrusion step. Fractions highlighted with dashed lines were further analyzed by UHPLC.

To separate the non-polar portion of the extract into additional fractions, CPC was performed with less polar solvent compositions than AZ-N, with AZ-T proving to be the appropriate choice. While the polar extract fraction was primarily found in fractions T-F2 (49.6% yield) and T-F3 (8.7% yield), the non-polar fraction could be divided into fractions T-F5 containing 13.2% of the extract and T-F6 with 27.5% (**Table 1**). Fractionation into T-F5 and T-F6 was primarily conducted on a visual basis, with T-F5 being yellow and T-F6 being green, as no UV signal was detectable with CPC-DAD (**Figure 1B**).

The use of the more polar solvent composition of the AZ-L system as compared to AZ-N allowed the separation of the polar extract fraction into two separate fractions: L-F2 with 31.5% yield and L-F3 with 25.0% yield (**Figure 1C**). Chromatograms of the CPC fractionations are shown in **Figure 1**. Fractions highlighted with dashed lines were selected for testing of their COX-2 inhibitory activity and further analyzed by UHPLC.

The Extract E70, the two nonpolar fractions T-F5 and T-F6, the polar fractions L-F2 and L-F3, and the fraction T-F2 were analyzed by UHPLC-CAD and -UV. For a better overview, the observed compounds

of the CPC fractions are displayed in the chromatogram of the extract E70 (**Figure 2**). Substance (1) at Rt = 15.92 min is tiliroside (supplementary data), already identified in our previous study [40]. The chromatograms of the individual CPC fractions are summarized in Supplementary Data (**Figure S1-5**).

The fraction T-F2 contained substances up to Rt = 19 min, while the fraction L-F2 primarily combined the substances up to Rt = 8 min. The fraction L-F3 contained less po-lar compounds up to Rt = 19 min, including tiliroside, which was not present in L-F2. In contrast, the fractions T-F5 and T-F6 collected molecules after Rt = 25 min, with fraction T-F5 containing substances up to Rt = 47 min and T-F6 containing substances from Rt = 33.5 min. The molecules present in the first half of the overlap region of fractions T-F5 and T-F6 were more prominent in T-F5, whereas the molecules in the second half of the over-lap region were more prominent in T-F6. With the aim of identifying the most active compounds, these five fractions were tested for their COX-2 inhibitory activity.

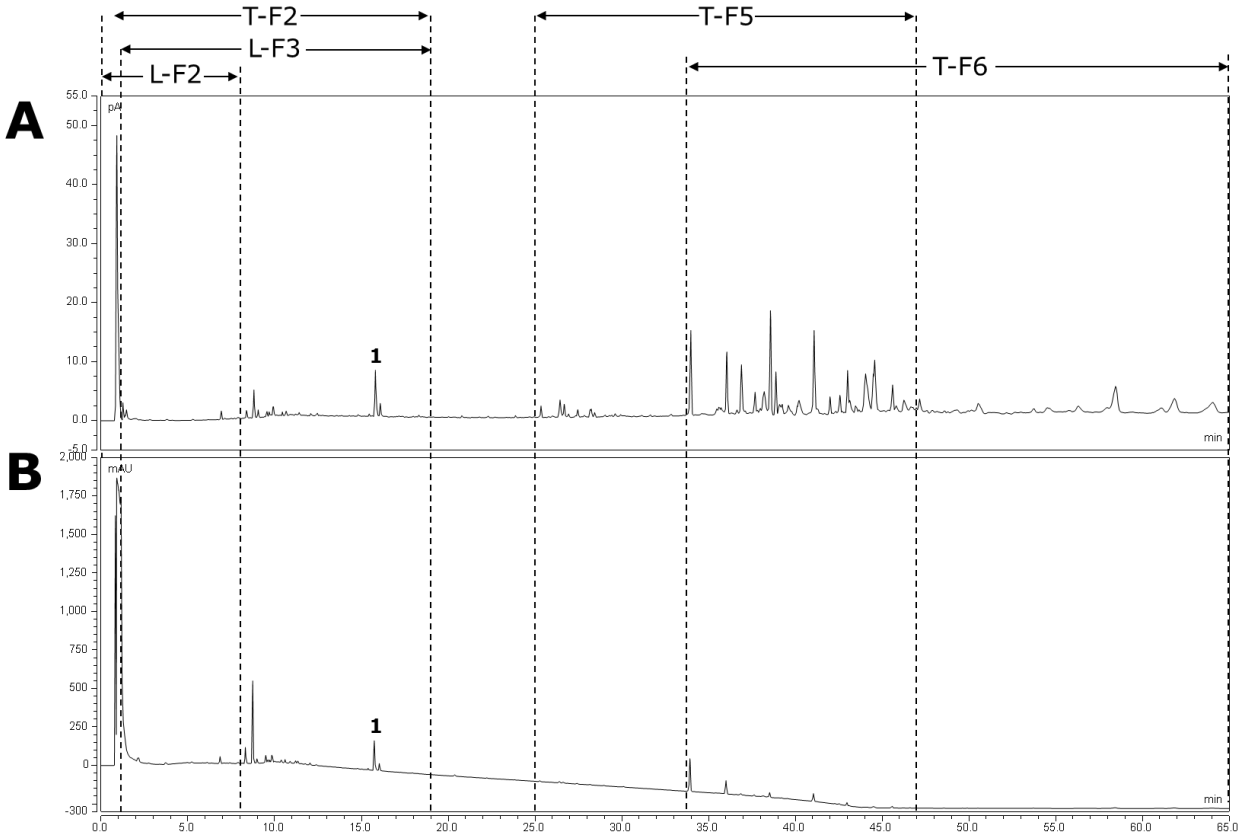

Figure 2: UHPLC chromatogram of Waltheria indica leaf extract E70 obtained at 70 °C with ethanol; the dashed lines show the observed compounds of the respective CPC fractions with (**A**) CAD and (**B**) DAD at 200 nm. The signal (1) at Rt = 15.81 min corresponds to that of tiliroside.

2.2. COX-2 Inhibitory Activity of CPC Fractions

The results of the relative COX-2 inhibition for the CPC fractions are summarized in **Figure 3**. For comparison, the positive control was set to 100% and the samples were calculated accordingly.

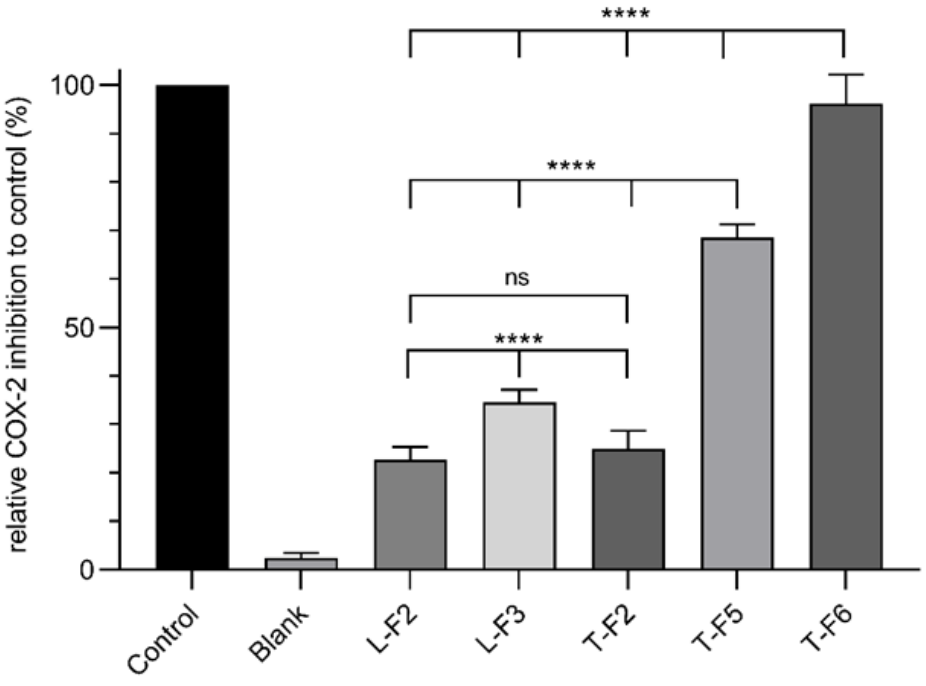

Figure 3. Relative COX-2 inhibition to control (%) of CPC fractions (20 µg/mL), blank (DMSO) and control set to 100% (0.5 µM celecoxib). Data points represent the mean value ± standard deviations of six samples. Statistically different expressions were calculated using one-way ANOVA. (****): $p < 0.0001$; (ns): not significant.

The generated CPC fractions exhibited a COX-2 inhibitory effect. The polar fractions L-F2, L-F3 and T-F2 showed COX-2 inhibitory activity levels of 22.8%, 34.6% and 25.1%, respectively. Among these CPC fractions, L-F3 showed the highest activity. This could be due to a higher enriched tiliroside concentration, which was either not present (L-F2) or represented a smaller part (T-F2) in the other fractions.

The nonpolar fractions T-F5 and T-F6 exhibited a significantly higher COX-2 inhibitory activity with 68.7 and 96.3%, respectively. These results indicate that the nonpolar fractions comprised compounds which are more potent than those in the polar fractions, supporting the similar observations of a recent study [40]. At that time, it was found that extracts produced with more polar solvents showed a weaker COX-2 inhibitory activity than extracts produced with non-polar solvents. Considering that the T-F5 and T-F6 fractions exhibited the highest activity, these CPC fractions were selected to elucidate the structure of the molecules responsible for the COX-2 inhibitory activity.

2.3. Structural Elucidation of the Molecules in Fraction T-F5 and T-F6

Three signals present in both the fraction T-F5 and T-F6 were identified by high resolution mass spectrometry (HPLC-MS/MS) with further verification by comparing frag-mentation data with the respective internal standard (**Figure S6-12**). The molecules identified and illustrated in **Figure 4** are the fatty acids (FA) alpha-linolenic acid (1), linoleic acid (2), and oleic acid (3), which, to the best of our knowledge, were here identified and re-ported for the first time in Waltheria indica leaf extracts.

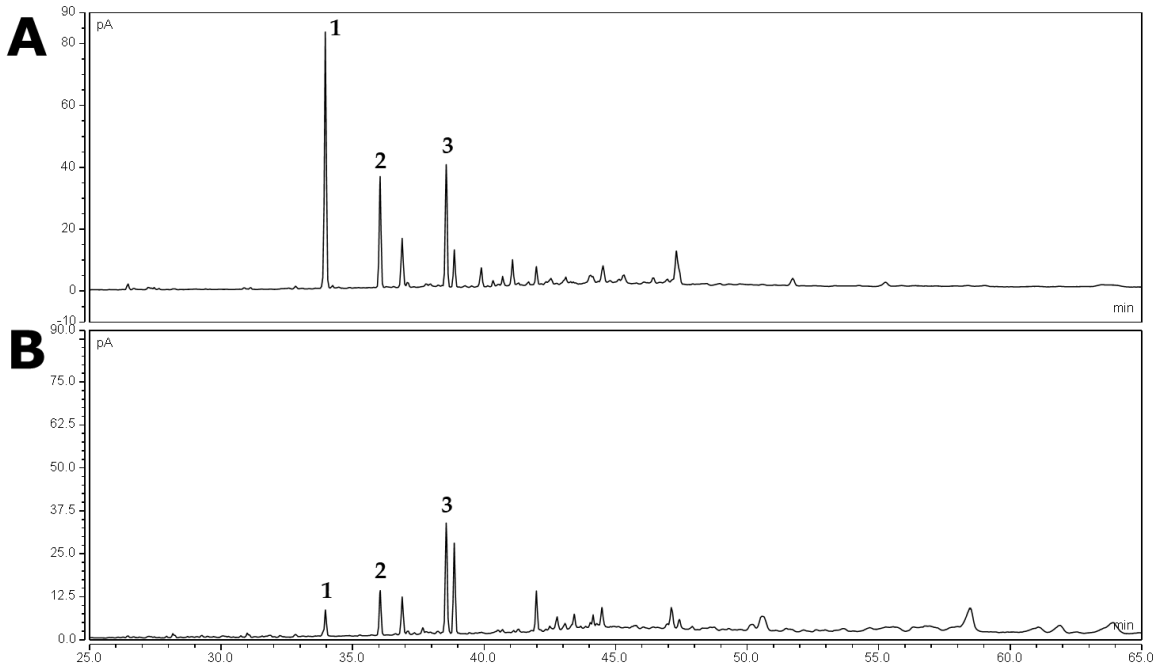

Figure 4. UHPLC-CAD chromatogram of the CPC fraction (A) T-F5 and (B) T-F6. The signal (1) at Rt = 33.91 min corresponds to alpha-linolenic acid, (2) that at Rt = 36.00 min corresponds to linoleic acid, and (3) that at Rt = 38.51 corresponds to oleic acid.

All three compounds have been reported to be COX-2 inhibitors, with alpha-linolenic acid (ALA) and linoleic acid (LA) being described as substantially more efficient than oleic acid (OA) [42,43]. The COX reaction produces prostaglandins by converting arachidonic acid (AA). However, this enzyme is capable of oxidizing other unsaturated fatty acids including alpha-linolenic acid and linoleic acid [44,45]. Structural determinations have revealed that, similarly to AA, these FA bind in elongated L-shaped conformations within COX-2, suggesting that their inhibitory activity originates in competing with arachidonic acid as substrates for COX-2 [43,46].

After having identified the three fatty acids, they were quantified in different Waltheria indica leaf extracts and in the CPC fractions T-F5 and T-F6.

2.4. Quantification of ALA, LA, OA in Extracts and CPC Fractions

With the aim of investigating the influence of the extraction parameters on the extraction of ALA, LA and OA from Waltheria leaves, their concentrations were quantified in extracts obtained by varying the extraction temperatures and solvents. Furthermore, their content was determined in the CPC fractions T-F5 and T-F6 as it was evident from the chromatograms prepared previously that these FA were not present in fractions L-F2, LF-3 and T-F2. The quantification of the FA further made it possible to verify, in further steps, whether their amount was sufficient for the COX-2 inhibitory activity in the samples. The concentrations (%, w/w) of ALA, LA and OA in the respective dry extract and in dried CPC fractions are summarized in **Figure 5**, with the extracts sorted in decreasing order by their contents of fatty acids. In addition, the extracted amounts of ALA, LA and OA from 1 g of dry plant material (mg/g plant) were calculated following Equation (2) and are listed in **Table 2**.

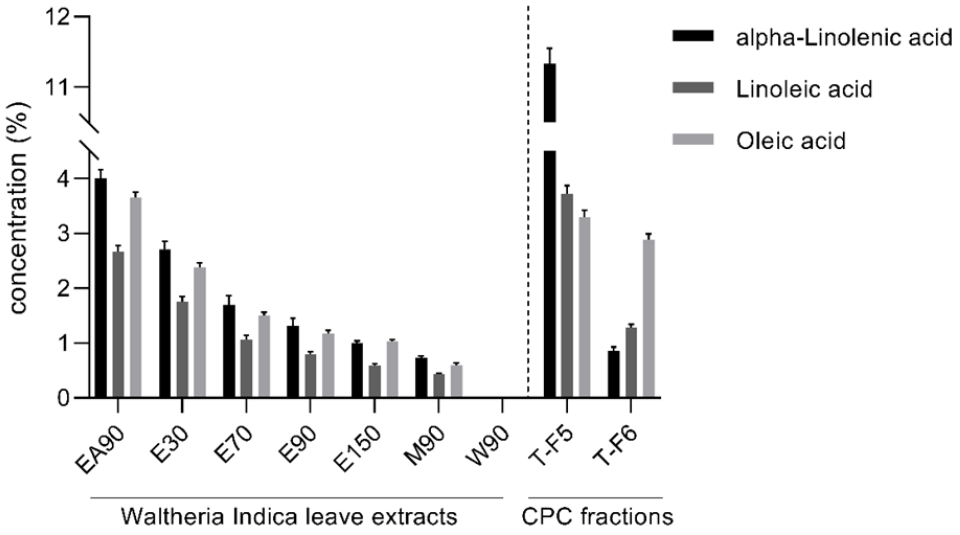

Figure 5. Concentration (%, w/w) of alpha-linolenic acid, linoleic acid and oleic acid in dried Waltheria indica leaf extracts obtained by varying extraction temperatures and solvents (left of dashed line) as well as CPC fractions T-F5 and T-F6 obtained from extract E70 (right of dashed line). Data points represent the mean value ± standard deviations of three samples. EA90, 90 °C ethyl acetate; E30–E70–E90–E150, 30–70–90–150 °C ethanol; M90, 90 °C methanol; W90, 90 °C water.

The extract obtained with ethyl acetate as a solvent at 90 °C (EA90) showed, with 4.0% ALA, 2.7% LA and 3.7% OA, the highest concentration of all three fatty acids among all extracts. The second highest concentration of the three fatty acids was observed in the extracts obtained after extraction with ethanol (E30-E150). The results showed that the in-crease in the extraction temperature from 30 °C (E30) up to 150 °C (E150) caused a reduction in the fatty acids in the extract (**Figure 5**). In contrast, the total amount of ALA, LA and OA extracted from 1 g plant material with ethanol increased with higher extraction temperature (**Table 2**). It can be inferred that a higher temperature contributes to the disruption of the plant cell wall, which enables the solvent to extract more lipids, while simultaneously extracting larger amounts of additional substances, resulting in a reduced content of fatty acids in the total extract. Extract E30 contained 2.7% ALA, 1.8% LA and 2.4% OA, whereas 1.0% ALA, 0.6% LA and 1.0% OA were present in extract E150. The concentration of the fatty acids in the extracts produced at 70 °C (E70) and 90 °C (E90) were 1.7% ALA, 1.1% LA and 1.5% OA, and 1.3% ALA, 0.8% LA and 1.2% OA, respectively. After extraction with methanol at 90 °C, the extract (M90) contained 0.7% ALA, 0.4% LA and 0.6% OA, the second lowest content of the fatty acids in the prepared extracts.

The investigations of the CPC fractions demonstrated that fatty acids were significantly more efficient enriched in the fraction T-F5 compared to T-F6. The concentrations of ALA, LA and OA in the fraction T-F5 were 11.3%, 3.7% and 3.3%, respectively. In fraction T-F6, the concentration was significantly lower with 0.9% ALA, 1.3% LA and 2.9% OA.

Based on the presented data, it is evident that ALA is the most abundant fatty acid in Waltheria leaves, followed by OA and LA. The results also highlight the influence of the polarity of the solvents. Solvents such as ethyl acetate and ethanol favor the accumulation of fatty acids in the extract more than the accumulation of polar solvents, methanol and water (**Figure 5**).

Table 2. Summary of extract yield (mg/g plant) and fatty acid content (mg/g plant) of Waltheria indica leaf extracts obtained with varying extraction temperatures and solvents. EA90, 90 °C ethyl acetate; E30–E70–E90–E150, 30–70–90–150 °C ethanol; M90, 90 °C methanol; W90, 90 °C water; n.d., not detectable. Values are means ± standard deviations of triplicate measurements.

Sample	EA90	E30	E70	E90	E150	M90	W90
Yield extract (mg/g plant)	29.4	15.5	74.0	108.9	170.0	210.1	225.4
ALA (mg/g plant)	1.18 ± 0.04	0.42 ± 0.02	1.26 ± 0.12	1.43 ± 0.16	1.70 ± 0.07	1.53 ± 0.09	n.d.
LA (mg/g plant)	0.78 ± 0.03	0.27 ± 0.01	0.79 ± 0.05	0.87 ± 0.04	1.00 ± 0.06	0.90 ± 0.04	n.d.
OA (mg/g plant)	1.07 ± 0.03	0.37 ± 0.01	1.11 ± 0.05	1.29 ± 0.06	1.75 ± 0.04	1.26 ± 0.08	n.d.

In addition, the results demonstrated that the highest extract yield was obtained with the most polar solvents (water > methanol > ethanol > ethyl acetate) operating at 90 °C, whereas increasing the temperature for ethanol extraction resulted in a higher extract yield (E30-E150). As a result of the overall higher extract yield with ethanol (E90) and methanol (M90) over ethyl acetate (EA90), more fatty acids were extracted per 1 g of dry plant material with these solvents (Table 2). The reason for the lower content of fatty acids in these extracts can be attributed to the fact that they contained larger amounts of additional polar substances, resulting in a dilution of the fatty acids in the total extract.

After determining the content of the individual fatty acids in the extracts and CPC fractions, the next step was to investigate the effect of the pure fatty acids and the obtained extracts in terms of their COX-2 inhibitory activity.

2.5. COX-2 Inhibitory Activity of ALA, LA, OA and Extracts

The COX-2 inhibitory activity of pure alpha-linolenic acid, linoleic acid and oleic acid was studied in the concentration range from 0.9 to 5.0 µM, which includes the concentration levels observed in the extracts and shown in **Figure 6**. Similarly, the extracts were analyzed for their COX-2 inhibitory activity and displayed in **Figure 7**.

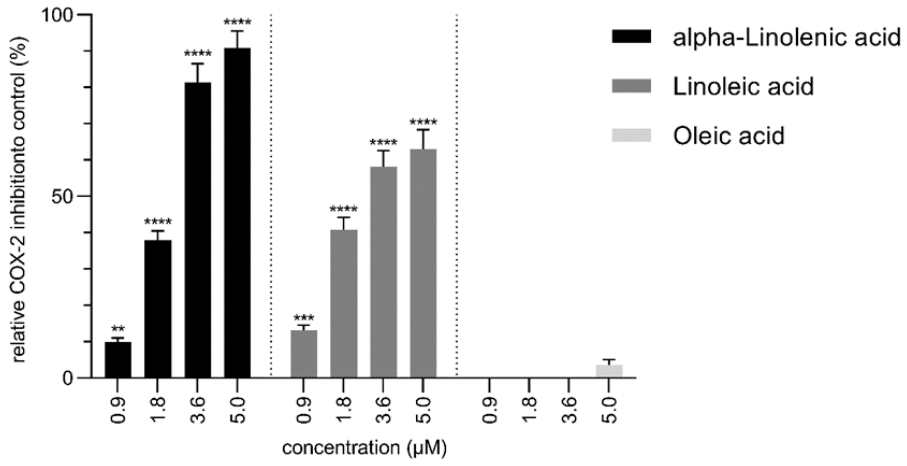

Figure 6. Relative COX-2 inhibition to control (%) of alpha-linolenic acid, linoleic acid and oleic acid with 0.5 µM celecoxib as control set to 100%. Data points represent the mean value ± standard deviations of six samples. Statistically different expressions were calculated using one-way ANOVA. (****): $p < 0.0001$; (***): $p = 0.0001$; (**): $p = 0.0029$ vs. blank.

The COX-2 inhibition of ALA and LA increased in a dose-dependent manner starting at a concentration of 0.9µM with 9.9% inhibition for ALA and 13.1% inhibition for LA and increasing up to 90.8% for 5.0µM ALA and 63.0% for 5.0µM LA. Oleic acid showed a measurable activity only at 5µM, with 3.5% activity. The results are consistent with previous reports that ALA and LA possess COX-2 inhibitory properties, whereas OA shows activity only at very high concentrations [42,43].

The studied extracts were sorted in decreasing order according to their ALA and LA contents, with EA90 containing the highest and W90 the lowest amount; they are plotted against their respective COX-2 inhibition activity in **Figure 7**. Knowing that oleic acid does not make a significant contribution to COX-2 inhibition at the concentrations present in the extracts, the following results will focus primarily on ALA and LA.

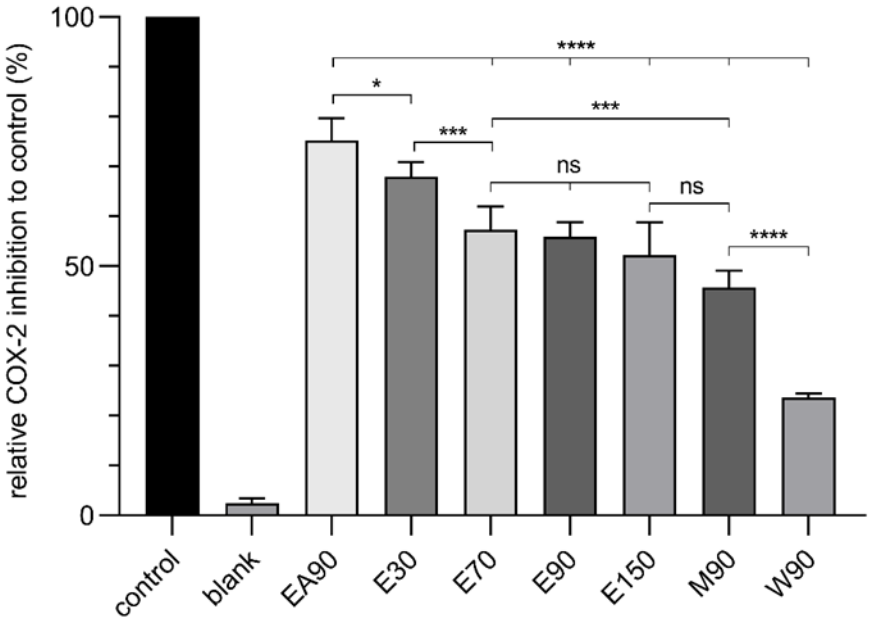

Figure 7. Relative COX-2 inhibition to control (%) of Waltheria indica extract solution (20 µg/mL), blank (DMSO) and control set to 100% (0.5 µM celecoxib). Data points represent the mean value ± standard deviations of six samples. Statistically different expressions were calculated using one-way ANOVA. (****): $p < 0.0001$; (***): $p = 0.0005$; (*): $p = 0.0442$; (ns): not significant.

The results indicate that the COX-2 inhibitory activity of the extracts declines with the decreasing of the fatty acids' concentrations. The observed relative COX-2 inhibitory activity was 75.4% for EA90, 67.9% for E30, 57.5% for E70, 56.0% for E90, 52.5% for E150, 45.8% for M90 and 23.7% for W90.

The extract EA90, with the highest concentration of ALA and LA content (4.0% ALA, 2.7% LA), corresponds to a concentration of 3.1µM pure ALA and 2.0µM pure LA in the assay. Based on the results in **Figure 6**, inhibition in the 60–80% range would be expected, with 3.0µM pure ALA alone matching the actual observed inhibition of 75.4%.

The observation that one fatty acid may be accountable for the complete COX-2 inhibition of the extract could not be made for the other extracts. Extract E30, for example, with ALA and LA concentrations in the assay corresponding to 2.0µM ALA and 1.3µM LA, would be expected to have an activity in the range between 30 and 40%. However, a COX-2 inhibitory activity of 67.8% was measured. The

comparison of the results of E70 and E150 further indicated that the concentration of fatty acids in these extracts does not translate 1:1 to the scaling of COX-2 inhibition of the extracts. E70 contained 1.7-fold higher ALA content and 1.8-fold higher LA content relative to E150, whereas the COX-2 inhibition of these extracts did not reflect this increase.

Based on these data, no definitive statement could be made regarding the exact contribution of the fatty acids to the COX-2 inhibition of the extracts. Therefore, the next step was to test for the presence of fatty acid mixtures (FAM) containing ratios of ALA, LA and OA in the respective extracts and examine their COX-2 inhibition.

2.6. COX-2 Inhibitory Activity of Fatty Acid Mixtures (FAM)

To investigate the accurate contribution of the fatty acids to the observed COX-2 inhibitory activity of the extracts, the formulated FAM of ALA, LA and OA, at a concentration and ratio identical to the corresponding extract, were prepared and analyzed for their COX-2 inhibitory activity. In addition, the contribution of the fatty acids to the total COX-2 inhibitory activity of the respective extract was calculated using Equation (6) and the results are summarized in **Table 3** with the extracts sorted in decreasing order according to their fatty acid contents, with EA90 containing the highest and W90 the lowest amount.

Table 3. Relative COX-2 inhibition to control (%) of Waltheria indica extract solution (20 µg/mL) and fatty acid mixture solution (20 µg/mL) with 0.5 µM celecoxib as control set to 100%. Data points represent the mean value ± standard deviations of six samples.

	EA90	**E30**	**E70**	**E90**	**E150**	**M90**	**W90**
Extract COX-2 inhibition (%)	75.4 ± 4.0	67.9 ± 2.7	57.5 ± 4.3	56.0 ± 2.6	52.5 ± 5.9	45.8 ± 3.1	23.7 ± 0.7
Corresponding FAM COX-2 inhibition (%)	27.6 ± 1.1	26.2 ± 1.5	23.9 ± 2.1	21.7 ± 1.9	18.5 ± 0.7	14.3 ± 0.9	0.6 ± 0.9
Contribution of FAM to COX-2 inhibition (%)	36.6 ± 2.5	38.6 ± 2.4	41.6 ± 3.1	38.8 ± 2.7	35.2 ± 3.2	31.3 ± 2.5	0

The results of the FAM indicated a similar trend to the one obtained from the corresponding extracts, showing a decline in activity as the concentration of fatty acids decreased. The contribution of fatty acids, when present in the extract, to COX-2 inhibition of the extract ranged from a minimum of 31.3% (M90) up to 41.6% (E70). These findings pro-vide clear evidence that the identified fatty acids, at their observed concentrations in the extracts, contribute substantially to the COX-2 inhibition observed with the respective Waltheria indica extracts and enable the preparation of extracts with enhanced anti-inflammatory properties by choosing extraction parameters that result in a higher ALA or LA presence in the extract.

To further characterize the compounds involved in the COX-2 inhibitory activity, the phytochemical compositions of the CPC fractions were further investigated.

Before that, it was assessed whether antioxidants play a role in COX-2 inhibition. The results showed no relationship between the COX-2 inhibitory activity of the samples and their corresponding antioxidant properties. The details are summarized in the Supplementary Material section for the purpose of completeness (**Figure S13**).

2.7. Phytochemical Composition of the CPC Fractions

The phytochemical composition of the CPC fractions was examined with the aim of characterizing further compounds with COX-2 inhibitory activity, given that they differ significantly in activity and composition. The quantifications of total phenols, and triterpenoid and steroidal saponins in each CPC fraction are summarized in Table 4. For a more complete overview, their ALA and LA contents, as well as the associated COX-2 in-hibition levels, are listed.

The highest phenolic contents were observed in the polar fractions L-F2, L-F3 and T-F2 with 288.3, 220.1 and 256.8 mg/g, respectively, which were significantly higher compared to the nonpolar fractions T-F5 and T-F6 with 24.2 and 21.0 mg/g. In contrast, the polar fractions contained significantly lower levels of triterpenoid saponins with 49.2, 77.5 and 67.5 mg/g for L-F2, L-F3 and T-F2, respectively, compared to T-F5 with 325.8 mg/g and T-F6 with 188.5 mg/g (Table 4). For steroidal saponins, fraction T-F6 contained the highest amount with 107.4 mg/g, followed by T-F5 with 30.8 mg/g, L-F3 with 26.5 mg/g, L-F2 with 19.3 mg/g and T-F2 with 18.8 mg/g (**Table 4**).

Table 4. Phytochemical composition in mg/g dry sample and relative COX-2 inhibition to control (%) of the Waltheria indica leaf extract fractions. Data points represent the mean value ± standard deviations of three samples. GAE = Gallic acid equivalent; OAE = Oleanolic acid equivalent; DE = Diosgenin equivalent; n.d., not detected.

CPC fraction	L-F2	L-F3	T-F2	T-F5	T-F6
Total phenols (mg GAE/g)	288.3 ± 4.1	220.1 ± 4.2	256.8 ± 3.1	24.2 ± 2.1	21.0 ± 1.7
Triterpenoid saponin (mg OAE/g)	49.2 ± 1.9	77.5 ± 2.1	67.5 ± 4.1	325.8 ± 4.8	188.5 ± 3.4
Steroidal saponin (mg DE/g)	19.3 ± 1.1	26.5 ± 0.9	18.8 ± 1.3	30.8 ± 1.5	107.4 ± 4.1
Alpha-linolenic acid (mg/g)	n.d.	n.d.	n.d.	113 ± 2.1	8.6 ± 0.7
Linoleic acid (mg/g)	n.d.	n.d.	n.d.	37 ± 1.5	12.9 ± 0.5
COX-2 inhibition (%)	22.8 ± 2.5	34.6 ± 2.4	25.1 ± 3.5	68.7 ± 2.5	96.2 ± 5.5

When considering the total phenol content in the polar fractions L-F2, L-F3 and T-F2 and their COX-2 inhibition, a correlation was not evident as L-F2, with the highest phenol content, had a lower COX-2 inhibitory activity compared to L-F3 with the lowest phenol content but the highest COX-2 inhibition. In the case of the triterpenoid- and steroidal saponin content, one could deduce that COX-2 inhibition increases with its increasing content in these fractions.

In the case of nonpolar fractions T-F5 and T-F6, the results revealed that although T-F6 contained lower content of triterpenoid saponins, ALA and LA, compared to T-F5 the observed COX-2 inhibitory activity

was significantly higher. This can be explained by the steroidal saponin content, which was 3.5 times higher in fraction T-F6, indicating that steroidal saponins in this case contributed predominantly to the COX-2 inhibitory activity.

Based on these data, it can be concluded that the steroidal and triterpenoid saponins, and ALA and LA, are among the prominent COX-2 inhibitory compounds in Waltheria indica extracts. With the aim of predicting the COX-2 inhibitory activity of the extracts, the next step was to attempt the development of mathematical models based on the composition and COX-2 inhibitory activity of the extracts studied.

2.8. COX-2 Inhibition Prediction Models

To predict the COX-2 inhibitory properties of different Waltheria indica extracts, two mathematical models (model A and B) were generated, considering steroidal and triterpenoid saponins as well as ALA and LA as positive contributors to the COX-2 inhibition. The phytochemical composition of the investigated extracts and their COX-2 inhibition activity served as the data basis (**Table S1**). Tiliroside, OA and phenols were not taken into consideration since experimental work indicated that they do not influence the COX-2 inhibitory activity at the concentration levels present in the extracts.

Model A was generated by considering, as a predictor variable for COX-2 inhibition activity, the weighted sum of the concentrations of the contributors in the extract. For each contributor, the slope of the linear regression line between the concentration of the contributor and COX-2 inhibition in the extracts served as the weighting factor. For ALA and LA, we observed a second-order polynomial relationship between their concentration and COX-2 inhibition activity. Thus, a linearization was applied to both variables, in this case a square root transformation, to obtain the slope of the linear regression line. The obtained weighting factors for the individual concentrations were 0.27 for triterpenoid saponins, 0.50 for steroidal saponins, 8.23 for the square root of ALA and 10.03 for the square root of LA. A linear regression line was then fit between the weighted sum of contributors (x-variable) calculated using Equation (7) and COX-2 inhibition activity (y-variable), leading to the equation $y = 0.257x + 20.911$.

Model B was generated using partial least squares (PLS) regression, which is a linear multivariate regression that is useful for analyzing data with many variables and few observations (as is in the present case: four variables with seven observations). The PLS regression considered as predictors (x-variables) the concentration of each positive contributor and the COX-2 inhibitory activity as a response variable (y-variable). Similar to Model A, ALA and LA concentration values were first square-root transformed to improve their linear relationship with COX-2 inhibitory activity. The obtained PLS model can be represented by the linear equation $y = 0.0693a + 0.1296b + 2.1269c + 2.5925d + 20.9035$.

Both models were used to generate a linearity plot, i.e., the scatter plot showing the relationship between the observed versus predicted COX-2 inhibitory activity values for the extracts, and to calculate the root mean square error (RMSE). The obtained linearity plots are depicted in **Figure 8**.

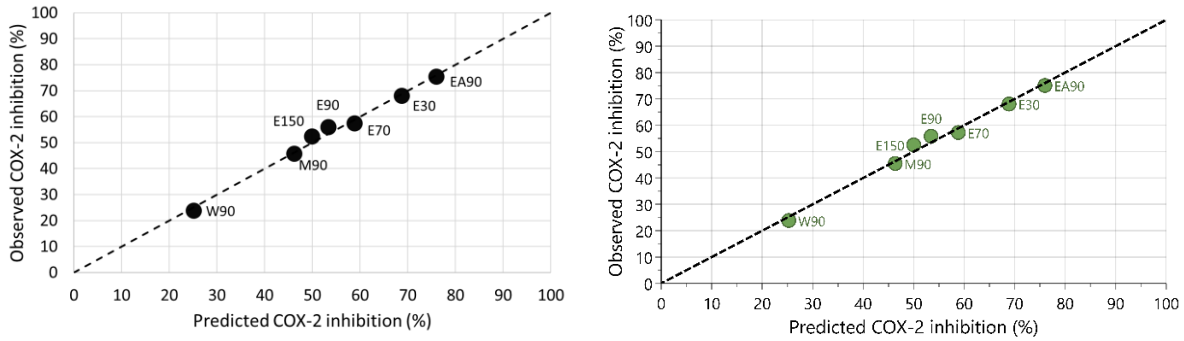

Figure 8. Graphs representing (A) model A linearity plot of observed versus predicted COX-2 inhibition of Waltheria indica extracts (RMSE = 1.6%) based on the obtained linear fit equation y=0.257x + 20.911 generated by the weighted sum of the contributors versus COX-2 inhibitory activity and (B) model B linearity plot of observed versus predicted COX-2 inhibition (RMSE = 1.6%) based on the generated linear fit equation y = 0.0693a + 0.1269b + 2.1269c + 2.5925d + 20.9035 by PLS regression. Dashed lines represent the line of identity (y=x). y = predicted COX-2 inhibition (%); x = weighted sum concentration of the contributors; a = triterpenoid saponin concentration; b = steroidal saponin concentration; c = square root concentration of ALA; d = square root concentration of LA.

The results demonstrate that the approach of using the weighted sum of the concentrations in model A led to similar results as the application of a multivariate regression method available in a commercial software carried out with method B. Applying the linear fit equation from model A or model B results in a similarly good fit with a RMSE = 1.6% COX-2 inhibition activity demonstrating a high correspondence between predicted versus observed values.

In both models, the data show that the coefficients of the contributors increase in the order LA > ALA > steroidal saponins > triterpenoid saponins, indicating that their contribution to COX-2 inhibitory activity in Waltheria indica extracts follows this order. It is also important to note that while these models provide good prediction results for Waltheria indica extracts, they are not necessarily applicable to CPC fractions or to pure substances. As it is important not to extrapolate, the concentration of the phytochemicals in the test samples should be within the concentration ranges of the extracts used for the models. While the individual fractions are simplified systems suitable for the identification of positive contributors to the COX-2 inhibitory activity, extracts are much more complex systems with a greater number of interactions between the individual components having an in-fluence on the potential biological activity. This becomes obvious when looking at the single substances without any interactions with other compounds in the extracts, where ALA showed a stronger contribution overall compared to LA (**Figure 6**). In the case of the CPC fractions where the concentrations of several phytochemicals were higher compared to their concentration in extracts, the data suggest that the steroidal saponins contribute the most (**Table 4**). In the event of the future discovery of additional substances that contribute positively to COX-2 inhibition and are present in sufficient concentrations in the extract, the mathematical models established here should be revisited and can be adopted accordingly.

Based on the proposed mathematical models, a more targeted development of extraction procedures is possible in order to obtain Waltheria indica extracts with improved anti-inflammatory properties. Furthermore, the transfer of the approach presented in this work to the prediction of other biological endpoints would be of great interest and should be considered in future studies.

3. Materials and Methods

3.1. Chemicals

Gallic acid, oleanolic acid, diosgenin, alpha-linolenic acid, linoleic acid and oleic acid (as reference standards) and Folin–Ciocalteu reagent, vanillin, perchloric acid, sulfuric acid, dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate, acetic acid, hydrochloric acid and anisaldehyde were obtained from Merck KGaA (Darmstadt, Germany). All other solvents and chemicals used were of analytical or HPLC grade. The working solutions were prepared immediately prior to measurement.

3.2. Plant Material

Waltheria indica L. plant was collected from Wageningen University & Research (Wageningen, Gelderland, Netherlands) and confirmed as Waltheria indica L. by Eurofins Genomics Europe Applied Genomics GmbH. Leaf samples were dried at a constant temperature of 40 °C for 48 h (Vacutherm, Thermo Scientific) and grounded into a fine powder before extraction.

3.3. Plant Extract Preparation

Plant extraction was performed by accelerated solvent extraction Dionex ASE 350 (Thermo Scientific, Waltham, MA, USA). For all extractions, 100 mL containers were charged with 10–12.5 g plant material mixed with diatomaceous earth (60-033854, Thermo Scientific, Waltham, MA, USA) as a neutral matrix to assure a 1:20 plant/solvent ratio. The ASE extraction was performed with 4 cycles in total, each cycle having a 6 min static time and a 160 s purge time at 1460 psi static pressure. The extraction temperature was 90 °C for all solvents and additionally 30, 70 and 150 °C for ethanol extraction. The obtained extracts were filtered through a 0.22 µm membrane (HPF Millex®, Merck Millipore, Burlington, MA,USA) and evaporated under vacuum at 60 °C. The extract yield (mg/g dry plant) was calculated using Equation (1) and the fatty acid yield extracted from 1 g plant material (mg/g dry plant) was calculated using Equation (2).

Yield extract = weight of dry extract/weight of dried plant material (1)

Yield fatty acid = concentration fatty acid in extract x yield extract (2)

3.4. Centrifugal Partition Chromatography

CPC separations were performed with a CPC-1000 (Gilson, Middleton, WI, USA) using a two-phase solvent system of the Arizona solvent family composed of heptane, ethyl acetate, methanol and water. Fractionation of the crude extract started with the solvent composition AZ-N (1:1:1:1, v/v). Subsequently, more polar solvent compositions (AZ-H (1:3:1:3, v/v), AZ-K (1:1:1:2, v/v) and AZ-L (2:3:2:3, v/v)) and non-polar solvent compositions (AZ-T (3:1:3:1, v/v) , AZ-R (2:1:2:1, v/v) and AZ-U (4:1:4:1, v/v)) were carried out. The fractionation of the extract was performed in the descending mode. The column was first filled with the stationary phase, then the apparatus was rotated at 1500 rpm and the CPC column was equilibrated with the mobile phase at a flow rate of 20 mL/min. After having reached the hydrodynamic equilibrium, a sample solution (50 mL, 3 mg/mL) was injected into the column. The

separation was performed at a flow rate of 20 mL/min and monitored with a DAD detector at 254 nm. The fractions were collected in 25 mL test tubes and evaporated under vacuum at 40 °C. The strategy for the combination of the fractions was based on the UV signal for the polar part of the plant. During the investigations, it became apparent that a number of compounds were not UV active. In particular, the fractionation of the non-polar part of the extract was partly realized on a visual basis, as the fractions showed color differences to some extent, with no detectable UV signal.

The CPC fraction yield (%, w/w) was calculated using Equation (3). The calculation of the non-polar part was primarily conducted on a visual basis, with fraction T-F5 being yellow and T-F6 being green, as no UV signal was detectable with CPC-DAD. The obtained fractions were dissolved in DMSO for further analysis (2 mg/mL).

Yield CPC fraction = weight of dry fraction/injected amount of dry extract × 100 (3)

3.5. Identification of Fatty Acids and UHPLC Analysis

The identification of alpha-linolenic acid, linoleic acid and oleic acid in the CPC fraction T-F5 was achieved using the Vanquish HPLC system coupled with a mass spectrometer (Q Exactive™ Hybrid Quadrupole-Orbitrap™, Thermo Scientific, Waltham, MA, USA) with H-ESI interface and negative ionization with 30 °C column temperature, 0.4 mL/min flow speed and 1 µL injection volume.

The separation was accomplished using a C-18 column (ThermoFisher™ Hypersil Gold™ aQ, 150 × 2.1 mm, 1.9 µm, Thermo Scientific, Waltham, MA, USA) and gradient elution with solvent A (water/formic acid 99.9/0.1 (v/v)) and B (acetonitrile/formic acid 99.9/0.1 (v/v)). The gradient started at 5% (v/v) B, was held constant for 1 min, ramped up to 50% over 20 min, ramped up to 98% over 20 min, and held constant for 25 min.

The HPLC-MS/MS chromatograms are summarized in the Supplementary Material section. The HPLC profile of the sample T-F5 showed three intense UV peaks at Rt = 33.91 min with a single charge state of MH- 277.2 g/mol, Rt = 36.00 min with MH- 279.2 g/mol and Rt = 38.51 min with MH- 281.2 g/mol. High resolution mass spectrometer fragmentation data confirmed the identification of alpha-linolenic acid, linoleic acid and oleic acid within the CPC fraction T-F5 (Figure S6-12).

The analysis of the CPC factions and extract samples as well as the quantification of alpha-linolenic acid, linoleic acid and oleic acid were carried out on a UHPLC (UltiMate 3000, Thermo Scientific, Waltham, MA, USA A) coupled with a CAD (Corona™ Veo™ RS, Thermo Scientific, Waltham, MA, USA) and a DAD (VANQUISH™ DAD HL, Thermo Scientific, Waltham, MA, USA). CAD detection was performed at 35 °C evaporation temperature and UV detection at 200 nm with 30 °C column temperature, 0.4 mL/min flow speed and 3 µL injection volume. The separation was accomplished with the identical column and method as used for the identification of the fatty acids by HPLC-MS/MS. The UHPLC-CAD chromatograms of the CPC fractions are summarized in the Supplementary Data (**Figure S1-5**). ALA, LA and OA concentrations present in the extracts and CPC fractions were calculated from a five data point calibration curve with the respective reference standard (0.010–0.140 mg/mL). The results were expressed in %, w/w.

3.6. COX-2 Inhibitory Activity

The ability of the CPC fractions and Waltheria indica extracts to inhibit COX-2 was determined using the fluorometric COX-2 specific inhibitor screening kit (BioVision, Zurich, Switzerland). The experimental protocol was followed according to the user manual. Fluorescence values (Ex/Em = 535/587 nm) of the samples were kinetically measured using a Tecan-Spark multimode microplate reader (Spark 20M, TECAN, Männedorf, Switzerland) at 25 °C for 10 min. Two appropriate points (T1 and T2) in the linear range of the plot were chosen, and the corresponding fluorescence values (RFU1 and RFU2) were obtained. The enzymatic assay was applied in the concentration range of 0.9–5 µM for the ALA, LA and OA reference standards and 20 µg/mL for the extracts and CPC fractions dissolved in dimethyl sulfoxide (DMSO). Each sample was analyzed six-fold; celecoxib (0.5 µM) was used as a positive control, while DMSO was used as a blank. The slope for all samples (S), including enzyme control (EC), was calculated by dividing the ΔRFU (RFU2–RFU1) values by the time ΔT (T2–T1). Subsequently, the percentage of relative COX-2 inhibition (RI) of the samples was first calculated using Equation (4):

$$\% \text{ relative COX-2 inhibition} = (\text{Slope of EC} - \text{Slope of S})/\text{Slope of EC} \times 100 \quad (4)$$

For a more accurate comparison of the results with subsequent experiments, the positive control was set to 100% and the percentage of relative COX-2 inhibition to the positive control was calculated according to Equation (5):

$$\% \text{ relative COX-2 inhibition to positive control} = \text{RI sample/RI positive control x } 100 \quad (5)$$

The contribution, in percentage, of the fatty acid mixtures to the total COX-2 inhibitory activity of the respective extract was calculated using Equation (6).

$$\text{Contribution} = \text{COX-2 inhibition FAM/COX-2 inhibition extract} \times 100 \quad (6)$$

3.7. Quantification of Phytochemicals

3.7.1. Phenol Quantification

For a reliable correlation between concentration of phenolic compounds and their activity, the total phenol content was determined using the Folin–Ciocalteu method with slight modifications [47]. The test solutions (1.0 mL, 1 mg/mL) were mixed with the Folin–Ciocalteu reagent (10 mL, previously diluted in water 1:10, v/v) and sodium carbonate (Na2CO3; 8.0 mL, 75 g/L). The tubes containing the solutions were vortexed for 15 s and incubated for 120 min at room temperature for color development. The absorbance was measured at 765 nm against a blank (methanol) using a Varian Cary 60 UV–VIS spectrophotometer (Agilent, Santa Clara, CA, USA). All assays were performed in triplicate. Gallic acid (0.0100–0.150 mg/mL) was used for calibration and the results were expressed as mg of gallic acid equivalents per gram of dry extract (mg GAE/g).

3.7.2. Triterpenoid Saponin Quantification

The triterpenoid content was determined based on the vanillin-perchloric acid method with slight modifications [48]. The test solution (200µL, 1mg/mL), in a 10 mL tube, was heated to evaporate the solvent and the solid was reconstituted in a vanillin-glacial acetic acid solution (300 µL, 5% w/v) and in

perchloric acid (1.0 mL, 70%). The sealed samples were heated for 45 min at 60 °C and afterwards cooled in an ice-water bath followed by the addition of glacial acetic acid (4.5 mL). The absorbance of the sample solutions was measured at 540 nm against a blank using a Varian Cary 60 UV–VIS spectrophotometer (Agilent, Santa Clara, CA, USA). The blank was treated identically with the exception that no vanillin was used. Oleanolic acid (0.0090–0.4000 mg/mL in methanol) was used for calibration and the results were expressed as mg of oleanolic acid equivalents per gram of dry extract (mg OAE/g).

3.7.3. Steroidal Saponin Quantification

The spectrophotometric quantification of the steroidal saponin content was based on the quantification of steroidal sapogenins method with minor modifications in which stable and reproducible results with several standards and without interference from sugars, sterols, fatty acid and vegetable oil were reported [49–51].

After the test solution (200µL, 1mg/mL), in a 10 mL tube, was heated to evaporate the solvent, the precipitate was dissolved in ethyl acetate (2.0 mL) and mixed with anisaldehyde/ethyl acetate solution (1.0 mL, 0.5%, v/v) and sulfuric acid (1.0 mL, previously diluted in ethyl acetate 1:1, v/v). The sealed samples were further incubated for 20 min at 60 °C and cooled afterwards in an ice-water bath followed by the addition of demineralized water (0.5 mL). The samples were incubated for 30 min before the absorbance of the sample solutions was measured at 430 nm against a blank using a UV–VIS spectrophotometer (Varian Cary 60, Agilent, Santa Clara, CA, USA). The blank was treated identically with the exception that no anisaldehyde was used. Diosgenin (0.0125–0.2000 mg/mL in methanol) was used for calibration and the results were expressed as mg of diosgenin equivalents per gram of dry extract (mg DE/g).

3.8. COX-2 Inhibition Prediction Models

Two mathematical models (model A and B) were generated to predict the COX-2 inhibitory properties of different Waltheria indica extracts, considering steroidal saponins (S) and triterpenoid saponins (T) as well as ALA and LA as positive contributors to the COX-2 inhibition. The phytochemical composition of the investigated extracts and their COX-2 inhibition activity served as the data basis.

Model A was generated by considering, as a predictor variable for COX-2 inhibition activity, the weighted sum of the concentrations of the contributors (Sw) in the extract. The weighting factor for each contributor i (Wi) was taken as the slope of the linear regression line between the concentration of the contributor (Ci) and the COX-2 inhibitory activity of the extracts. For ALA and LA, a second-order polynomial relationship between their concentration and COX-2 inhibition activity was observed. To obtain the slope of the linear regression line, and thus, the corresponding Wi values, a linearization was applied to both variables—in this case, a square root transformation. The weighted sum (Sw) in model A of the concentrations of the contributors in the extract was calculated following Equation (7):

$$S_W = W_S \times C_S + W_T \times C_T + W_{ALA} \times \sqrt[2]{C_{ALA}} + W_{LA} \times \sqrt[2]{C_{LA}} \quad (7)$$

The weighted sum (x-variable) of each extract was plotted versus the respective COX-2 inhibitory activity (y-variable) to obtain the linear regression line. The obtained Wi was 0.27 for triterpenoid saponins, 0.50

for steroidal saponins, 8.23 for the square root of ALA and 10.03 for the square root of LA. The so obtained equation y = 0.257x + 20.911 was used to generate the predicted versus observed COX-2 inhibitory activity linearity plot and to calculate the root mean square error (RMSE)

Model B was generated using PLS regression, which is a linear multivariate regression, computed with SICMCA® (version 17.0), a multivariate data analysis software application (Sartorius AG, Goettingen, Germany). The model used, as predictor variables (x-variables), the concentration of the four positive contributors (considering the square root values for ALA and LA), and the COX-2 inhibitory activity was used as the dependent variable (y-variable). Before model fitting, variables were mean centered and scaled to unit variance. Model optimization was based on internal validation by considering leave-one-sample-out cross-validation (using a 95% confidence level). Based on the latter, the final PLS model dimensionality (i.e., the number of significant PLS components) was one, and all the predictor variables were found to be significant to model the response of interest (and thus, were included in the PLS model).

3.9. Statistical Analysis

All results are expressed as means ± standard deviation (SD). Determinations of phytochemical and fatty acid concentrations were performed in triplicate and the determination of COX-2 inhibition was performed with n=6. Statistical data processing was carried out by one-way analysis of variance (ANOVA) to assess the statistical significance of the observed differences. The statistical analysis was carried out using GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

4. Conclusions

In this study, we showed that the activity-guided approach led to the identification of alpha-linolenic acid, linoleic acid and oleic acid, which were identified and reported for the first time in Waltheria indica leaf extracts. The study revealed that they are responsible for up to 41% of the COX-2 inhibition observed in the respective Waltheria indica extract, with ALA and LA proving to be the major contributors, of the three fatty acids, to COX-2 inhibition. Through additional phytochemical analyses of the extract fractions, substances from the group of steroidal saponins and triterpenoid saponins were also identified as positive contributors to the COX-2 inhibitory activity. The identification of substances contributing to COX-2 inhibition enabled the successful development of mathematical models to predict the COX-2 inhibition of Waltheria indica extracts. Based on the mathematical models, it can be inferred that the contribution of the different substances in the Waltheria indica extracts follows the order linoleic acid > alpha linolenic acid > steroid saponins > triterpenoid saponins. These findings enable a more targeted development of extraction processes to obtain Waltheria indica extracts with superior anti-inflammatory properties.

Supplementary Materials: The following are available online, Figure S1: UHPLC chromatogram of CPC fraction L-F2 with (A) CAD and (B) DAD at 200 nm. Figure S2: UHPLC chromatogram of CPC fraction L-F3 with (A) CAD and (B) DAD at 200 nm. Figure S3: UHPLC chromatogram of CPC fraction T-F2 with (A) CAD and (B) DAD at 200 nm. Figure S4: UHPLC chromatogram of CPC fraction T-F5 with

(A) CAD and (B) DAD at 200 nm. Figure S5: UHPLC chromatogram of CPC fraction T-F6 with (A) CAD and (B) DAD at 200 nm. Figure S6: UHPLC-UV chromatogram at 200 nm of (A) CPC fraction T-F5 (2 mg/mL) and (B) alpha-linolenic acid reference standard (0.05 mg/mL), (C) linoleic acid (0.05 mg/mL), (D) oleic acid (0.05 mg/mL). Figure S7: MS data of (A) peak at Rt = 33.91 with a single charge state of MH- 277.2 g/mol and (B) alpha-linolenic acid reference standard. Figure S8: Fragments (A) of 277.2 g/mol signal in fraction T-F5 and (B) of alpha-linolenic acid reference standard. Figure S9: MS data of (A) peak at Rt = 36.00 with a single charge state of MH- 279.23 g/mol and (B) linoleic acid reference standard. Figure S10: Fragments (A) of 279.2 g/mol signal in fraction T-F5 and (B) of linoleic acid reference standard. Figure S11: MS data of (A) peak at Rt = 38.51 with a single charge state of MH- 281.2 g/mol and (B) oleic acid reference standard. Figure S12: Fragments (A) of 281.2 g/mol signal in fraction T-F5 and (B) of oleic acid reference standard. Figure S13: IC50 values (µg/mL) of extracts (left of dashed line) and CPC fractions (right of dashed line) required to inhibit 0.1 mM DPPH. Table S1: Phytochemical composition in mg/g dry sample and relative COX-2 inhibition to control (%) of the Waltheria Indica leaf extracts.

Author Contributions: Conceptualization, M.T., C.M.K. and J.v.H; methodology, M.T.; software, M.T.; validation, M.T., J.v.H., A.S. and C.C.; formal analysis, M.T.; investigation, M.T.; resources, J.v.H.; data curation, M.T.; writing—original draft preparation, M.T.; writing—review and editing, J.v.H., C.C., A.S., J.H. and C.M.K; visualization, M.T.; supervision, J.v.H., J.H. and C.M.K; project administration, J.v.H.; funding acquisition, J.v.H. All authors have read and agreed to the pub-lished version of the manuscript.

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Data Availability Statement: Data are available from the authors upon reasonable request.

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Conflicts of Interest: Andrew Salazar, Joerg von Hagen and Christophe Carola are associated with Merck KGaA (Darmstadt, Germany). The funders had no role in the design of the study, in the collection of data or in the analyses.

Sample Availability: Samples of Waltheria Indica L. leaves are available from the authors.

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SUPPLEMENTARY MATERIAL

2.1 Centrifugal partition chromatography fractionation

The chromatograms of the individual CPC fractions are summarized in Figure S1-5. The CPC fraction L-F2 primarily combines the substances up to Rt = 8 min (Figure S1).

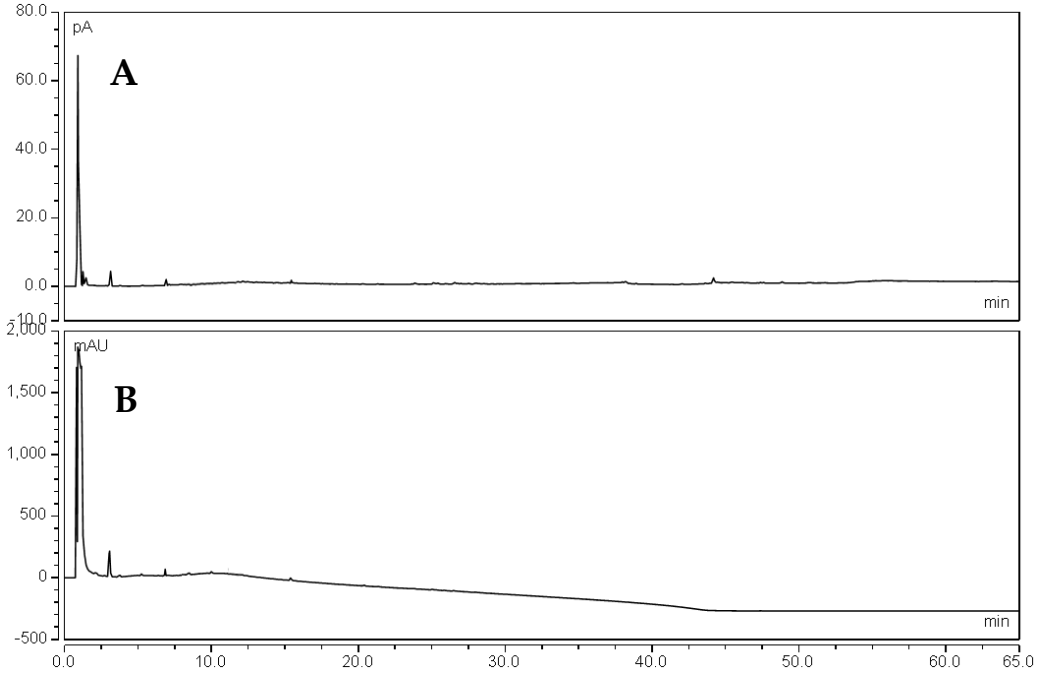


Figure S1: UHPLC chromatogram of CPC fraction L-F2 with **(A)** CAD and (**B**) DAD at 200 nm.

The fraction L-F3 contained less polar compounds up to Rt = 19 min including tiliroside, which was not present in L-F2 (Figure S2).

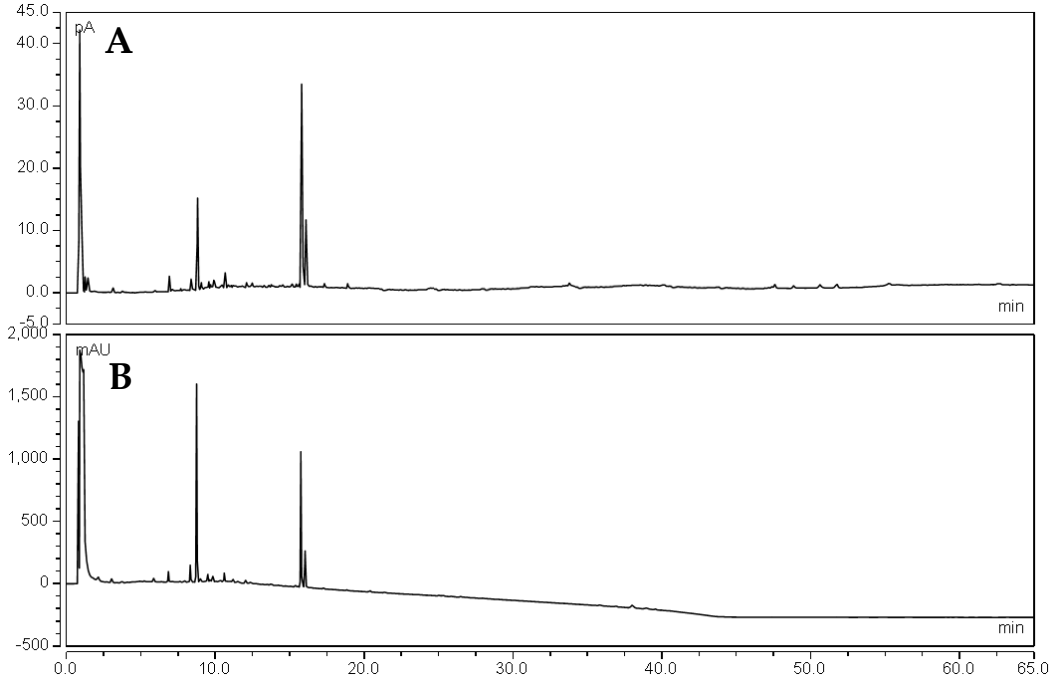


Figure S2: UHPLC chromatogram of CPC fraction L-F3 with **(A)** CAD and (**B**) DAD at 200 nm.

The fraction T-F2 contained substances between Rt=0 and Rt = 19 min (Figure S3).

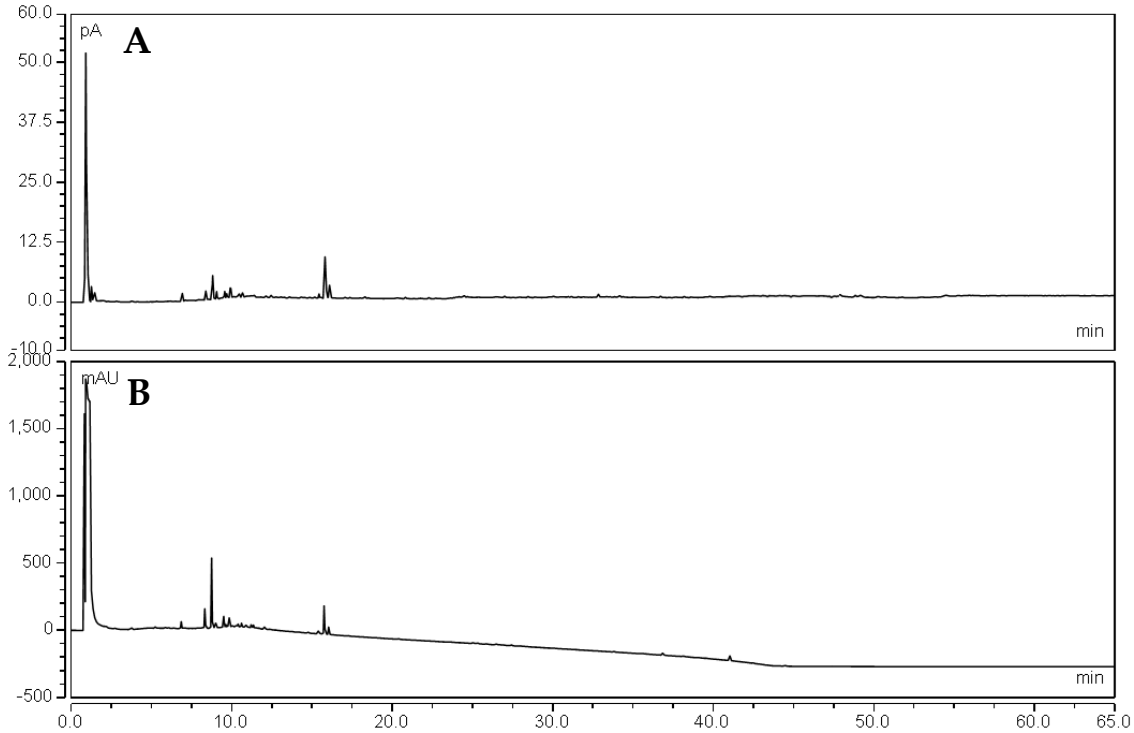


Figure S3: UHPLC chromatogram of CPC fraction T-F2 with **(A)** CAD and (**B**) DAD at 200 nm.

The fraction T-F5 collect molecules after Rt = 25 min up to Rt = 47 min (Figure S4).

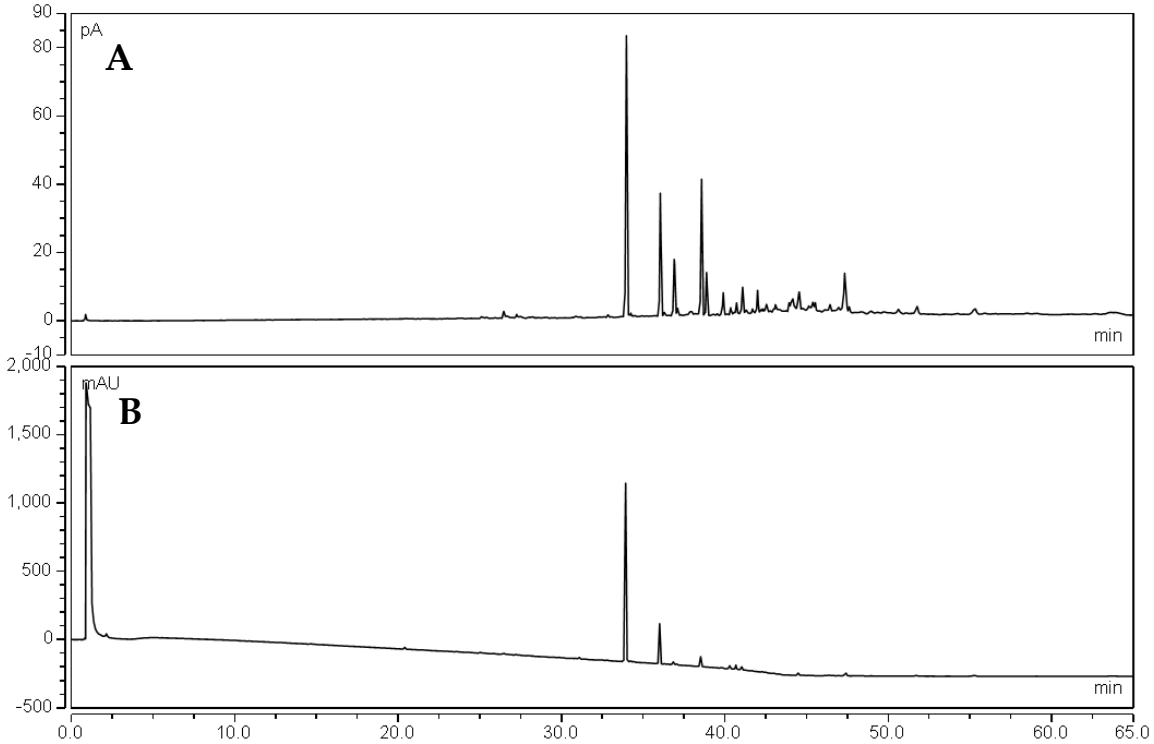


Figure S4: UHPLC chromatogram of CPC fraction T-F5 with **(A)** CAD and (**B**) DAD at 200 nm.

The CPC fraction T-F6 collect molecules from Rt = 33.5 min (Figure S5).

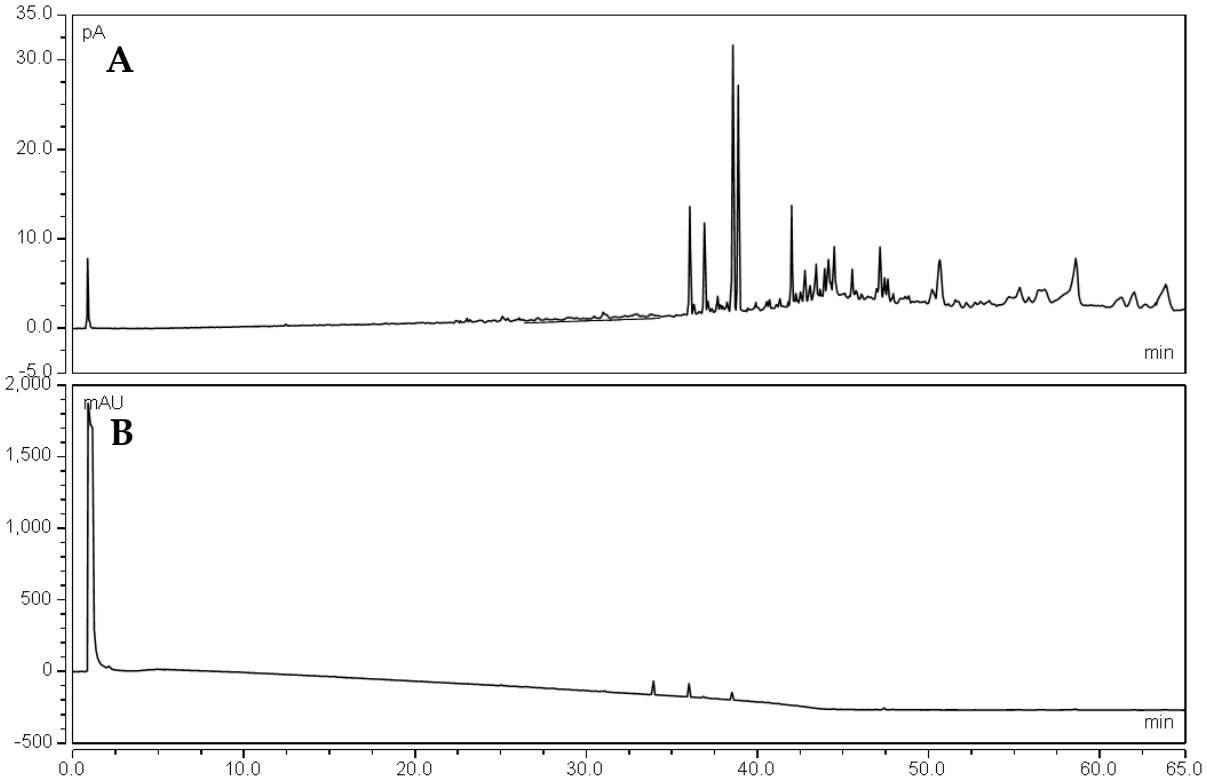


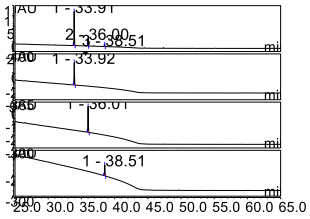
Figure S5: UHPLC chromatogram of CPC fraction T-F6 with **(A**) CAD and (**B**) DAD at 200 nm.

2.3 Identification of fatty acids in CPC fraction T-F5

The identification of the fatty acids alpha-linolenic acid, linoleic acid and oleic acid in the CPC fraction T-F5 was carried out with Vanquish HPLC system coupled with high resolution mass spectrometer (Q Exactive™ Hybrid Quadrupol-Orbitrap™, Thermo Scientific™, USA) with H-ESI interface and negative ionization at 30 °C column temperature, 0.4 ml/min flow speed and 1 µL injection volume.

The separation was accomplished using C-18 column (ThermoFisher™ Hypersil Gold™ aQ, 150 x 2.1 mm, 1.9 µm) and gradient elution with solvent A: water/formic acid 99.9/0.1 (v/v) and B: acetonitrile/formic acid 99.9/0.1 (v/v). Gradient started at 5% (v/v) B holding for 1 min, ramping up to 50 % over 20 min, ramping up to 98 % over 20 min and holding for 25 min.

UV-HPLC chromatogram at 200 nm of the fraction T-F5 showed three intense UV peaks at Rt = 33.91 min, R_t = 36.00 min and R_t = 38.51 min (Figure S6, A), which corresponds to reference standards of alpha-linolenic acid (Figure S6, B), linoleic acid (Figure S6, C) and oleic acid (Figure S6, D).



B

C

D

Figure S6: UHPLC-UV chromatogram at 200 nm of **(A)** CPC fraction T-F5 (2 mg/mL) and reference standards **(B)** alpha-linolenic acid (0.05 mg/mL), **(C)** linoleic acid (0.05 mg/mL), **(D)** oleic acid (0.05 mg/mL).

MS data show peak at R_t = 33.91 min with a single charge state of MH- 277.2 g/mol (Figure S7, A), which corresponds to that of alpha-linolenic acid reference standard (Figure S7, B)

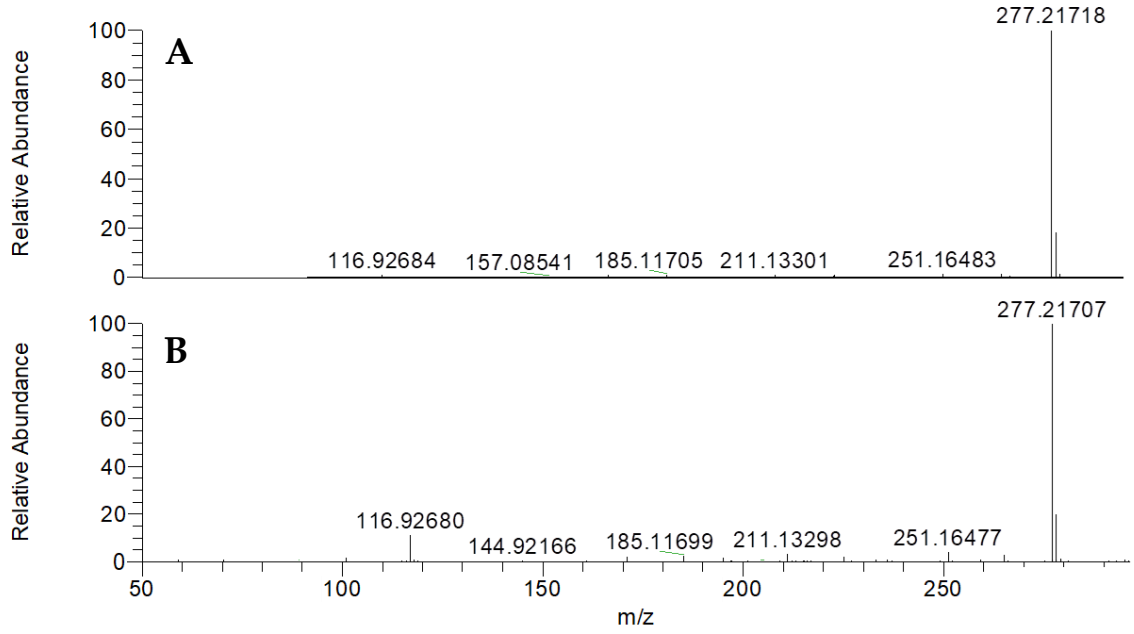


Figure S7: MS data of **(A)** peak at R_t = 33.91 with a single charge state of MH- 277.2 g/mol and **(B)** alpha-linolenic acid reference standard.

Fragments of the 277.2 g/mol signal in fraction T-F5 (Figure S8, A) correspond to alpha-linolenic acid reference standard fragments (Figure S8, B).

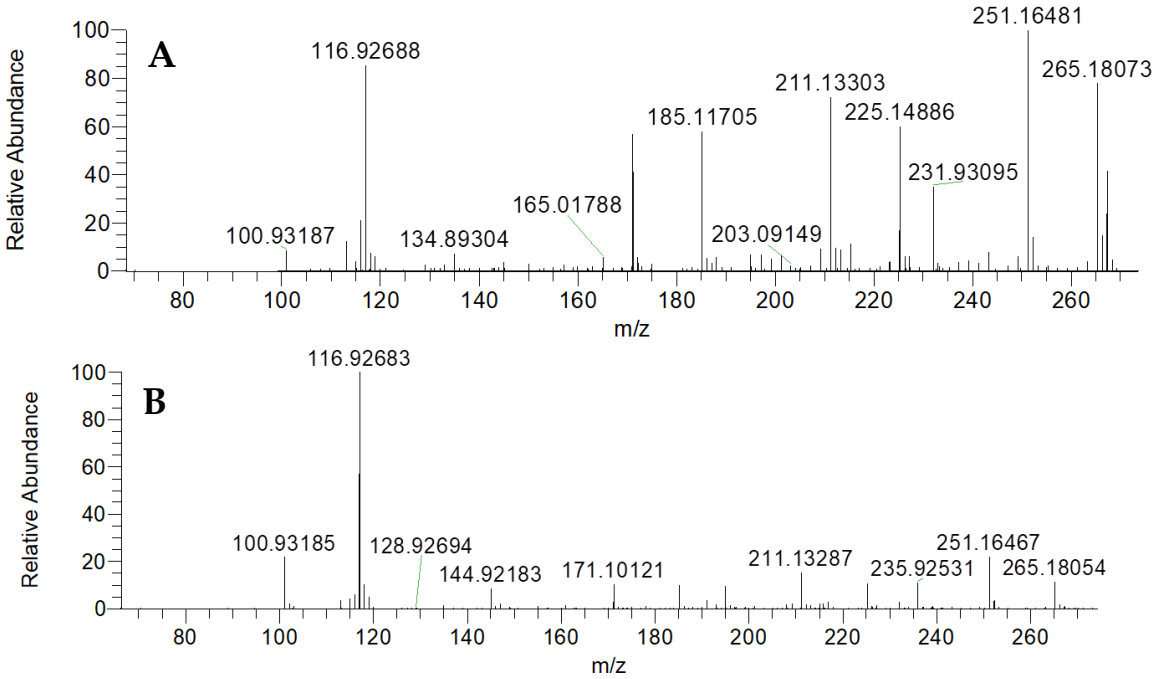


Figure S8: Fragments **(A)** of 277.2 g/mol signal in fraction T-F5 and **(B)** of alpha-linolenic acid reference standard.

MS data for the peak at R_t = 36.00 min with a single charge state of MH- 279.2 g/mol (Figure S9, A), which corresponds to that of linoleic acid reference standard (Figure S9, B)

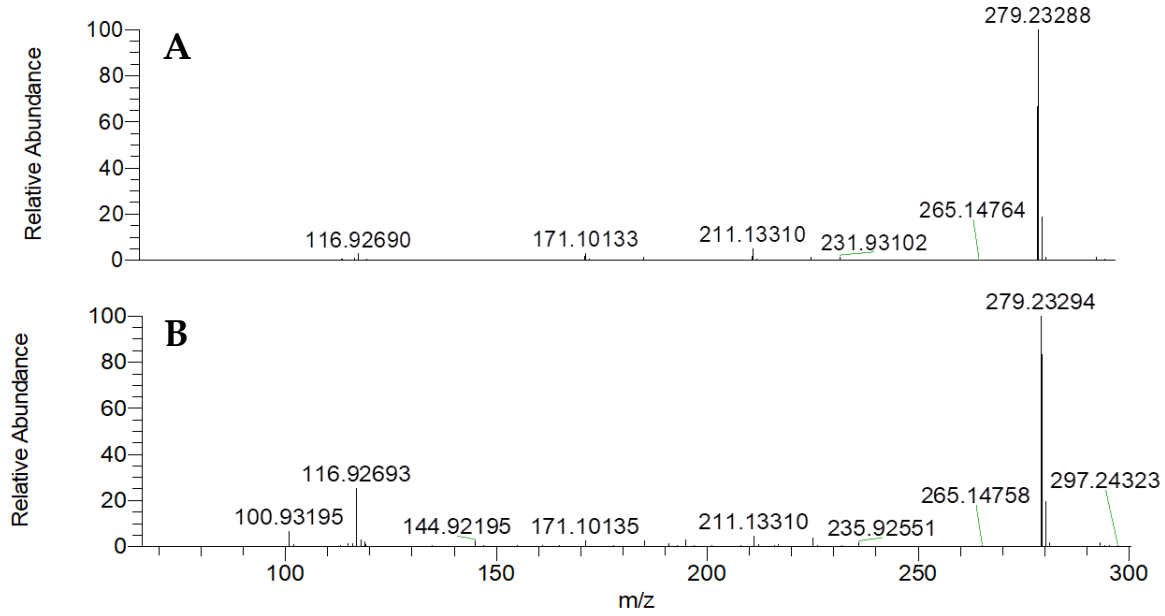


Figure S9: MS data of **(A)** peak at R_t = 36.00 with a single charge state of MH- 279.23 g/mol and **(B)** linoleic acid reference standard.

Fragments of the 279.2 g/mol signal in fraction T-F5 (Figure S10, A) correspond to linoleic acid reference standard fragments (Figure S10, B).

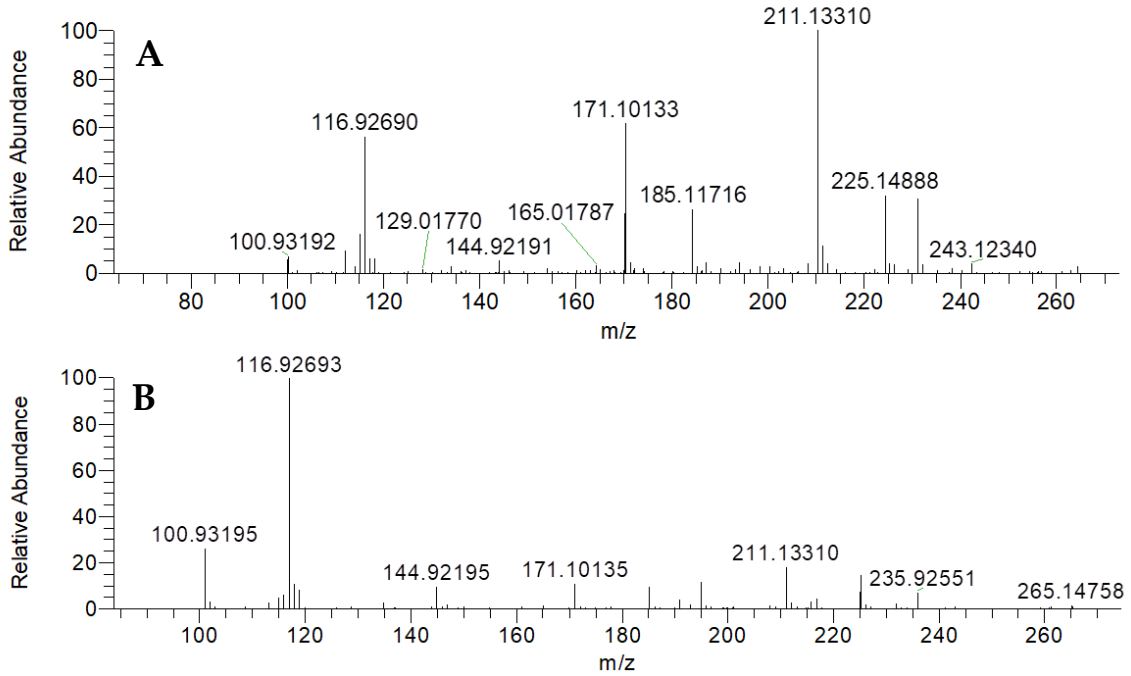


Figure S10: Fragments **(A)** of 279.2 g/mol signal in fraction T-F5 and **(B)** of linoleic acid reference standard.

MS data for the peak at R_t = 38.51 min with a single charge state of MH- 281.2 g/mol (Figure S11, A), which corresponds to that of oleic acid reference standard (Figure S11, B)

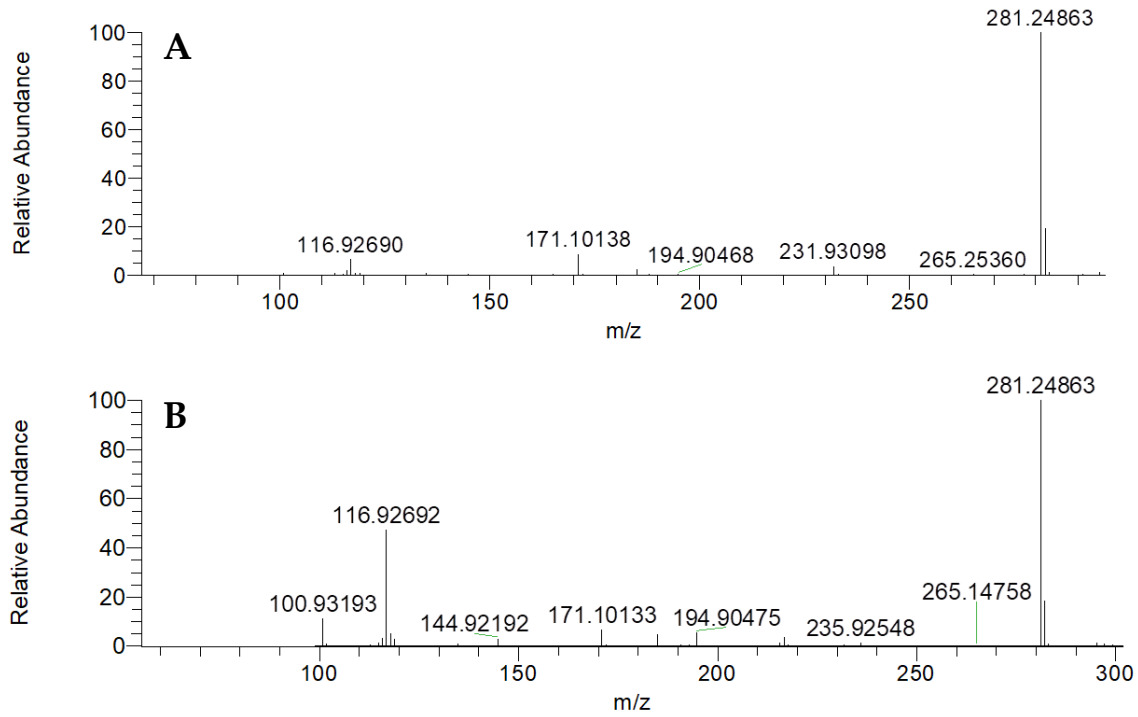


Figure S11: MS data of **(A)** peak at R_t = 38.51 with a single charge state of MH- 281.2 g/mol and **(B)** oleic acid reference standard.

Fragments of the 281.2 g/mol signal in fraction T-F5 (Figure S12, A) correspond to oleic acid reference standard fragments (Figure S12, B).

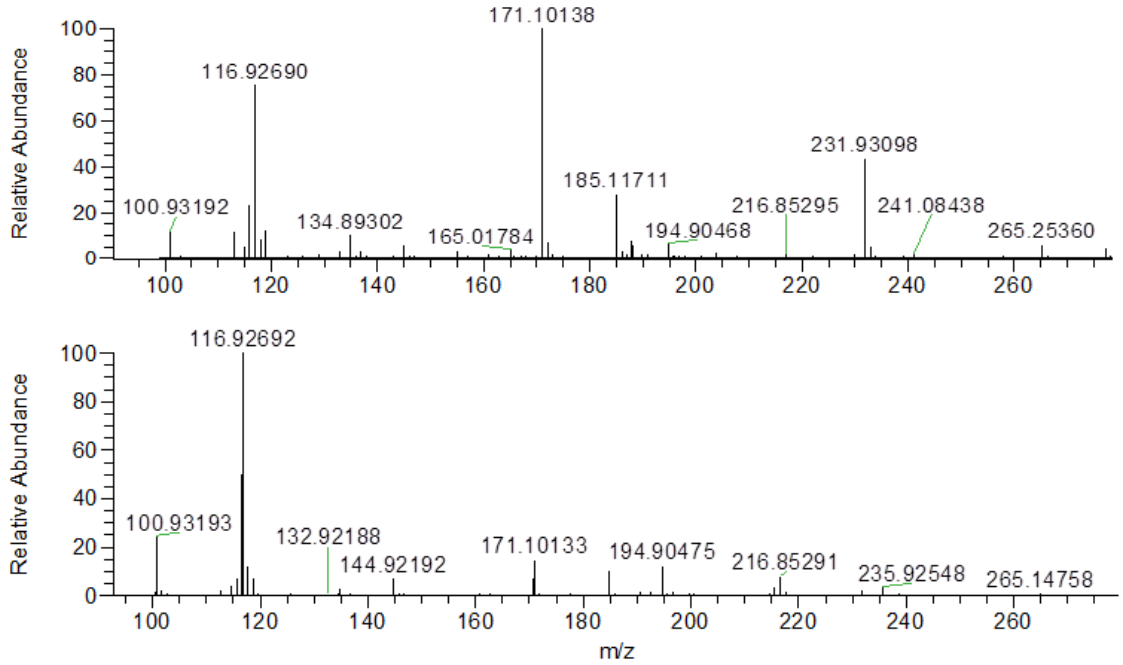


Figure S12: Fragments **(A)** of 281.2 g/mol signal in fraction T-F5 and **(B)** of oleic acid reference standard.

2.6 Antioxidative potential of extracts and CPC fractions

The antioxidant potential of the extracts and CPC fractions was assessed based on their concentration required to inhibit 50 % (IC_{50}) of 0.1 mM 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH). The IC_{50} values were calculated using a calibration curve with five data points with the respective sample (0.050 - 0.50 mg/mL) after determining the inhibition of the 0.1 mM DPPH solution for the respective concentration. Sample solution (1.0 mL) for the respective concentration was mixed with DPPH solution (9 mL; 0.112 mM dissolved in ethanol) resulting in sample (S) reacting with 0.10 mM DPPH. After 60 min reaction time the reduction of DPPH radicals was measured by the decrease in absorption (A) at 515 nm using a UV–VIS spectrophotometer (Varian Cary 60, Agilent). Ethanolic 0.1 mM DPPH solution was used as control sample (CS). The inhibition of 0.1 mM DPPH for the respective sample was calculated following equation S1.

$$\%\ \text{Inhibition of 0.1 mM DPPH} = (A(Cs)-A(s)) / A(Cs) \times 100 \qquad (S1)$$

After determining the DPPH inhibition for each sample following equation 1, the IC_{50} values were calculated based on the calibration curves generated from these data and summarized in Figure S13. All assays were performed in triplicate and results expressed as IC_{50} in mg/mL.

The obtained results demonstrated that among the extracts, EA90 had lowest antioxidant potential with IC_{50} = 108 µg/mL and E70 had the highest with IC_{50} = 37 µg/mL. Extracts E30, E90, E150, M90, and W90 exhibited significantly higher antioxidant potential relative to EA90 with IC_{50} values of 48, 41, 45, 43, and 44 µg/mL, respectively. Among the CPC fractions, the polar fractions L-F2, L-F3, and T-F2 with IC_{50} values of 24.5, 31.5, and 25.4 µg/mL, respectively, showed significantly stronger antioxidant potential than the nonpolar fractions T-F5 and T-6 with IC_{50} values of 617 and 362 µg/mL, respectively.

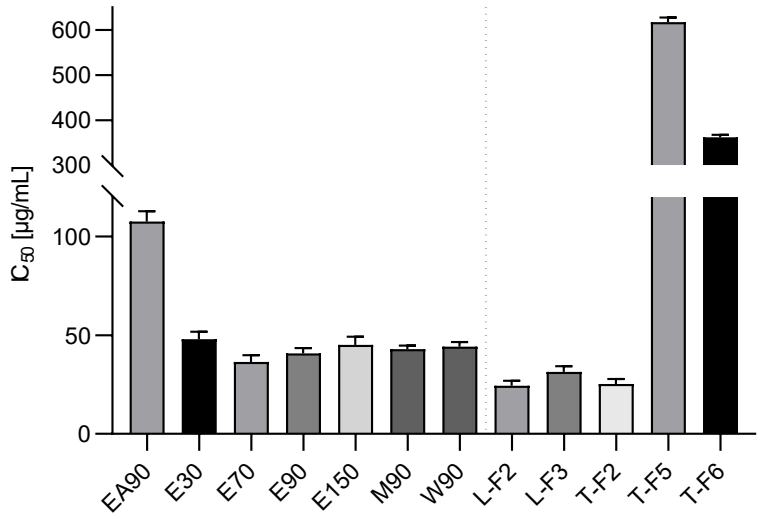


Figure S13: IC_{50} values (µg/mL) of extracts (left of dashed line) and CPC fractions (right of dashed line) required to inhibit 0.1 mM DPPH. Data points represent the mean value ± standard deviations of three samples (n = 3).

Given that samples with the highest COX-2 inhibition activity (EA90 for extracts and T-F5, T-F6 for CPC fractions) possess the weakest antioxidant capacity, it became evident that the observed COX-2 inhibition was not driven by antioxidants.

2.8 COX-2 inhibition prediction model

To predict the COX-2 inhibitory properties of different *Waltheria indica* extracts, two mathematical models (model A and B) were generated, considering Steroidal- and Triterpenoid saponins as well as ALA and LA as positive contributors to the COX-2 inhibition. The phytochemical composition of the investigated extracts and their COX-2 inhibition activity served as the data basis (Table S1)

Table S1: Phytochemical composition in mg/g dry sample and relative COX-2 inhibition to control (%) of the Waltheria Indica leaf extracts. GAE=Gallic acid equivalent; OAE=Oleanolic acid equivalent; DE=Diosgenin equivalent. EA90, 90 °C ethyl acetate; E30-E70-E90-E150, 30-70-90-150 °C ethanol; M90, 90 °C methanol; W90, 90 °C water; n.d., not detectable. Values are means ± standard deviations of triplicate measurements.

Sample	Triterpenoid-saponins [mg OAE/g]	Steroidal-saponins [mg DE/g]	Alpha-Linolenic acid [mg/g]	Linoleic acid [mg/g]	COX-2 inhibition [%]
EA90	195.0 ± 3.6	113.6 ± 2.7	40.1 ± 1.5	26.7 ± 1.1	75.3 ± 4.0
E30	214.8 ± 2.3	85.1 ± 1.3	27.1 ± 1.5	17.6 ± 0.9	68.0 ± 2.7
E70	182.8 ± 5.3	62.3 ± 3.5	17.0 ± 1.6	10.7 ± 0.7	57.3 ± 4.3
E90	143.6 ± 2.5	57.7 ± 1.5	13.1 ± 1.4	8.0 ± 0.4	56.0 ± 2.6
E150	120.1 ± 3.6	59.2 ± 1.4	10.0 ± 0.4	5.9 ± 0.3	52.3 ± 5.9
M90	106.0 ± 3.9	53.0 ± 0.8	7.3 ± 0.4	4.3 ± 0.2	45.7 ± 3.1
W90	47.3 ± 2.1	7.4 ± 0.7	0	0	23.7 ± 0.7

Identification of tiliroside in Waltheria Indica extract

Identification of tiliroside in *Waltheria Indica* extracts were carried out on Vanquish HPLC system coupled with Thermo Fusion™ Orbitrap Tribrid™ mass spectrometer (Thermo Scientific™) with H-ESI interface and positive ionization. Separation was accomplished using C-18 column (Thermo Acclaim™ 120, 100 x 4.6 mm, 5 µm) and gradient elution with solvent A: water/acetonitrile/trifluoroacetic acid 95/5/0.125 (v/v/v) and B: water/acetonitrile/trifluoroacetic acid 5/95/0.125 (v/v/v). Gradient started at 7% (v/v) B, ramping up to 28 % over 15 min, ramping up to 56 % over 5 min, further to 95 % over 5 min, held for 3 min and ramping down to 7 % over 8 min. UV detection was performed at 315 nm, 25 °C column temperature, 1.5 ml/min flow speed and 10 µL injection volume. UV-HPLC profile of *Waltheria Indica* extract obtained with ethanol at 90 °C show an intense UV peak at R_t = 14.47 min (**Figure S14**), which corresponds to tiliroside standard (data not shown).

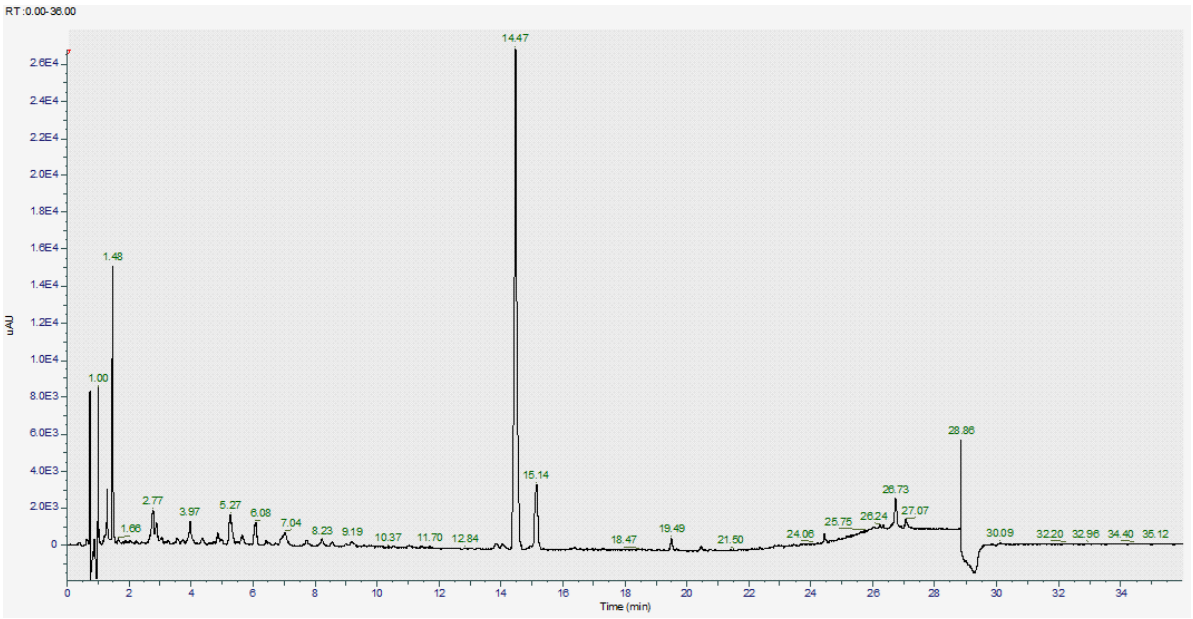


Figure S14: UV-HPLC profile at 315 nm of *Waltheria Indica* extract obtained with ethanol at 90 °C.

MS data in **Figure S15** of the peak at R_t = 14.47 show a single charge state MH+ 595.1432 g/mol and MNa+617.1249 g/mol, which corresponds to that of tiliroside.

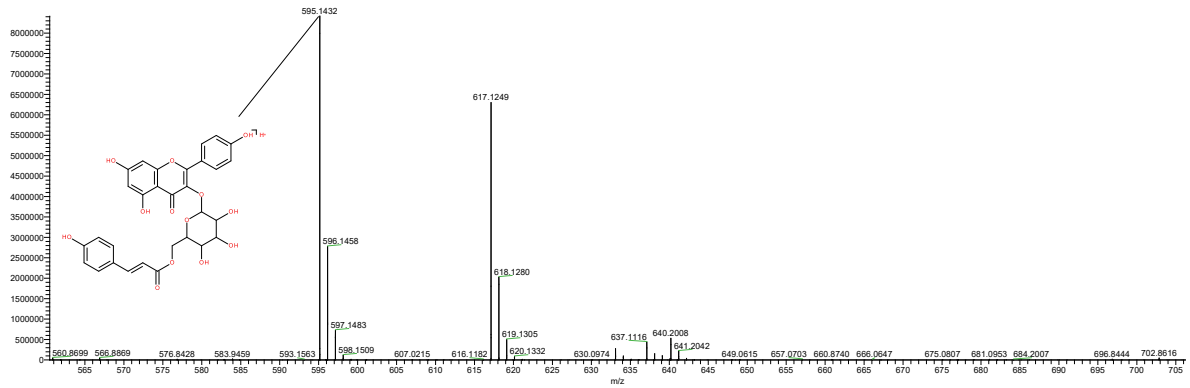


Figure S15: MS data of the peak at R_t = 14.47.

Fragments of 594.1432 g/mol signal correspond to tiliroside fragments (**Figure S16**).

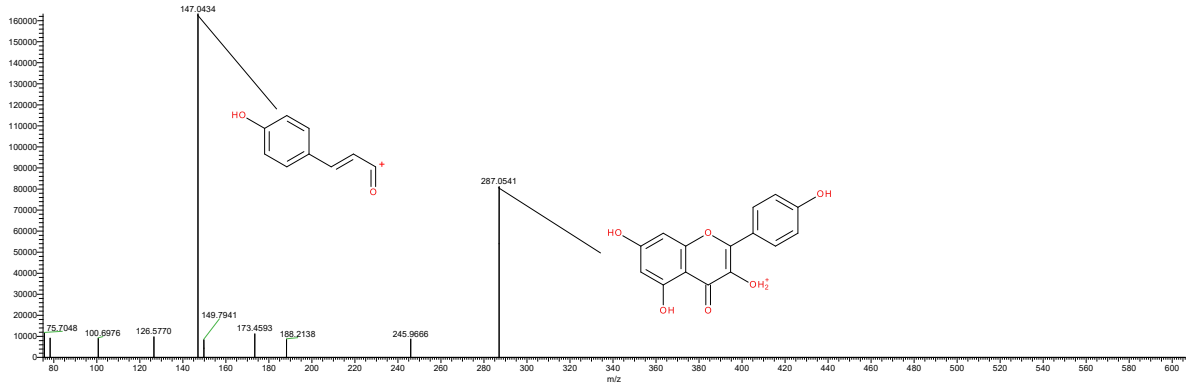


Figure 16: Fragments of 595.1432 g/mol signal with their corresponding structures.

6. Summary and Discussion

6.1. Summary and Discussion

Waltheria indica L. is one of the most widely used traditional medicinal plants with anti-inflammatory properties whose extracts have demonstrated COX-2 inhibitory activity [1-3]. However, the compounds responsible for the activity are regularly merely assumed and, in most cases, unknown or assigned to molecules whose content in the extract is insufficient or not known at all. The activity of *Waltheria indica* is often associated with the presence of flavonoids such as tiliroside [4,5]. However, their content remains unknown, and it is uncertain whether their concentration is sufficient for the observed efficacy.

Influence of extraction parameters on tiliroside content and its COX-2 inhibitory activity

The first step was to establish the extent to which tiliroside contributes to the COX-2 inhibitory activity of *Waltheria indica* leave extracts. For this purpose, different extraction parameters were applied in an attempt to maximize the content of tiliroside in the final extract. ASE extraction technique was selected as the primary extraction method given that extraction with ASE is significantly accelerated and ensuring constant conditions during extraction with high reproducibility of the results. Moreover, studies demonstrated that the phytochemical composition in the generated extracts remains similar to extracts obtained with classical extraction methods performed at similar conditions [6]. Given that the solubility of the target compounds in the solvent used for the extraction is the most relevant aspect of the entire extraction process, water, methanol, ethanol and ethyl acetate were applied as solvents during the extraction to cover a wide range of polarities. The extraction temperature was set to 90 °C for all solvents to achieve faster diffusion rates and improved solubility of the substances from the plant matrix. For extractions with ethanol, the influence of the extraction temperature was investigated in more detail, with extractions at 30, 70, 90 and 150 °C. Ethanol is classified as an environmentally preferable green solvent as it is produced by fermenting renewable sources including sugars and starches, which, along with water, makes it the preferred solvent for the extraction of natural substances [7,8]. In addition, by altering the extraction temperature, two goals were achieved simultaneously. First, extracts were generated with different concentrations of tiliroside as well as phytochemicals, which allowed to draw conclusions for more optimal extraction conditions of the respective constituent. Secondly, the extracts produced exhibited different COX-2 inhibitory activities, enhancing the establishment of relationships between the individual phytochemicals and their contribution to the activity.

Initially, the influence of the extraction parameters were examined with regard to the total dry extract yield in order to draw subsequent conclusions concerning alterations in the content of the various compounds in the extracts. The results demonstrated that operating at constant temperature the highest extract yield was obtained using more polar solvents as extractant (water > methanol > ethanol > ethyl acetate). Applying water as extractant resulted in a 7.8 times higher extract yield by comparison to ethyl acetate. This can be attributed to the circumstance that plants consist of large amounts of polar compounds e.g. sugars, glycosides or phenolic acids which are preferentially extractable with polar solvents [9,10]. The investigations on the influence of temperature during the extraction process clearly demonstrated that the employment of higher temperatures resulted in a significant increase of the

extract yield. Increasing the extraction temperature from 30 °C to 150 °C resulted in an extract yield increase by a factor of 10.7. It can be inferred that the use of elevated temperatures increased the solvent capacity to solubilize the phytochemicals, while simultaneously reduced the solvent viscosity enabling enhanced penetration of the plant matrix resulting in faster diffusion rates. In addition, higher temperature contributes to disrupting solute-matrix interactions and enhancing the extraction of phytochemicals from the plant matrix, leading to an increase in extract yield. These findings will be considered in further discussions.

The analysis of the tiliroside content utilizing HPLC revealed that extracts obtained at 90 °C contained the highest amount of tiliroside when using the medium-polar solvent ethanol, followed by methanol and significantly lower quantity when extracted with polar (water) or non-polar (ethyl acetate) solvent. These findings match the conclusions from other studies, where extracts generated from other plants with ethanol contained the highest levels of tiliroside [11]. The investigations into the influence of temperature on the extraction with ethanol resulted in three main observations. Firstly, elevating the temperature caused in an ever-increasing amount of tiliroside extracted per gram of plant material. Secondly, the content of tiliroside in the dry extract increased up to 90 °C extraction temperature and was significantly lower at 150 °C. Thirdly, it become evident that at 30 °C, no tiliroside was extracted from the plant (0.009 mg of tiliroside per gram of dry plant at 30 °C vs. 3.4 mg/g at 150 °C). These findings suggest that tiliroside present in *Waltheria indica* leaves is not readily accessible and the solvent does not sufficiently penetrate the cell wall and the lipid bilayer cell membrane to dissolve tiliroside. Providing sufficient energy, e.g. increased temperature, is therefore essential to disrupts the tiliroside–matrix interactions to achieve a high tiliroside content in the final extract. The fact that the content of tiliroside in the extract drops again when extracting at 150 °C, even though a higher amount was extracted per gram of plant, can be explained as a result of other unknown substances being extracted to a greater extent leading to a dilution of tiliroside in the total extract. Considering the obtained findings, it can be stated that the aim of generating an extract with a high content of tiliroside was realized with ethanol at 90 °C.

To verify that COX-2 inhibitory activity of the *Waltheria indica* extracts correlates with the tiliroside content, their activity was examined utilizing an enzyme-based COX-2 assay. Initially, the activity of pure tiliroside reference standard was investigated in a concentration range from 1.5 to 150 µM. Tiliroside demonstrated a concentration-dependent inhibition of COX-2 activity, with the lowest concentration showing significant activity at 15 µM. These findings are of importance as no studies of tiliroside activity in enzymatic COX-2 assays have been reported in the literature allowing a comparison with other compounds screened for their activity by means of enzymatic COX-2 assays. Subsequent to the studies of pure tiliroside reference standard, the COX-2 inhibitory activity of the extracts obtained varying solvents and extraction temperatures were investigated. Here, all extracts exhibited COX-2 inhibitory properties to a certain extent. However, the correlation between tiliroside content and COX-2 inhibitory activity could not be confirmed for the extracts. Extracts with the highest tiliroside content did not exhibit the highest COX-2 inhibition, in contrast highest inhibitory activity was found in extracts with low tiliroside content. It was found that even in the extract with the highest content of tiliroside, the concentration corresponded merely to 0.8 µM pure tiliroside in the assay. These findings revealed that the observed COX-2 activity of the extracts was not primarily derived from the contained tiliroside. The results further

revealed that the extracts obtained with different solvents have greater COX-2 inhibitory properties in the order ethyl acetate > ethanol > methanol > water, suggesting that the substances with higher COX-2 inhibitory activity belong to the non-polar compound classes. In addition to the observed influence of solvent polarity, an influence of temperature was noted. The results indicate that with increasing temperature, the COX-2 inhibitory activity of the extracts decrease. Several aspects are involved in this phenomenon. On the one hand, more substances are extracted from the plant as the temperature rises, which leads to the assumption that the most active substances are readily extractable. The extraction of further substances has the consequence that readily extracted compounds are diluted in the final extract and a lower activity observed. On the other hand, the active compounds could be thermally labile substances and an increase in temperature might accelerate their degradation. However, the observation that an extract prepared with ethyl acetate at 90 °C exhibits comparable activity to an extract obtained with ethanol at 30 °C contradicts the assumption of the thermal instability. The results strongly indicate that the phytochemicals with the highest COX-2 inhibitory activity are hydrophobic and readily available in the plant matrix.

Influence of extraction parameters on phytochemicals

In parallel to investigations regarding tiliroside, the influence of the extraction parameters on the composition of the extracts on their phytochemical composition were determined with regard to the content of total phenols, total alkaloids, triterpenoid- and steroidal-saponins. The goal was to investigate whether and to what extent the COX-2 inhibitory activity correlates with the content of these compounds in the extracts.

The composition of the phytochemicals of the extracts revealed that, except for ethyl acetate as solvent, the total phenol concentrations vary marginal using different solvents or temperatures. The content of phenols in the extract generated with ethyl acetate was 3 times lower compared to the extracts generated with other solvents at the same temperature. Considering the diversity of phenols with widely varying polarity, it can be concluded that the phenols in *Waltheria indica* leaves consist primarily of polar and moderately non-polar species. The observation that with increasing extraction temperature the content of phenols in the extract remains almost unchanged supports the assumption that phenols are present in all areas of the plant matrix. However, the comparison of extract COX-2 inhibition activity with the respective phenol content showed no discernible correlation with results providing no evidence that phenols are major contributor to the COX-2 inhibitory activity of the extracts. This becomes evident when comparing the extract generated with water as solvent to the one generated with ethyl acetate. The extract generated with water yielded a higher total phenol content by a factor of 2.8, however the COX-2 inhibitory activity was lower by factor 4.2.

In contrast to the relatively high content of phenols observed in *Waltheria indica* leave extracts, the analyses of the total alkaloid content in the extracts revealed low or undetectable concentrations. No alkaloids are detected in the extracts generated with water or ethyl acetates as solvent, indicating that the alkaloids present in *Waltheria Indica* leaves are generally soluble in polar protic mediums (ethanol and methanol). In addition to the solvent impact, an influence of temperature was also observed. The findings demonstrated that elevating temperature during extraction with ethanol as solvent, the extracted

alkaloid content increased significantly. These results suggest that the alkaloids in the leaves of *Waltheria indica* constitute a minor proportion and are presumably stronger chemically bonded to the plant matrix, which hinders their extraction. Furthermore, a comparison of the total alkaloid content and the COX-2 inhibitory activity of the extracts provided no apparent evidence of a correlation. This is illustrated by the observation that no alkaloids were detected in ethyl acetate extract, while the extract exhibited the highest COX-2 inhibitory activity and in addition, by the findings that although the alkaloid content extracts increased at elevated extraction temperature, the activity of the extracts declined. These findings are important as there have been no studies on the COX-2 inhibitory activity of alkaloids found *Waltheria indica* leaves. Studies investigating alkaloids in *Waltheria indica* extracts, primarily from the roots and aerial parts, led to the isolation and characterization of more than 20 alkaloids. Two of them, waltherione A and C, demonstrated anti-inflammatory activity via inhibiting NF-κB, while the research focus for the other alkaloids was towards their anti-trypanosomal and antifungal activity [12-14]. With the results obtained in the framework of this thesis, it can be stated that the COX-2 inhibitory activity of the extracts were not the result of the presence of the alkaloids in leaves of *Waltheria indica*.

Alongside phenols and alkaloids, saponins were found to be among the most prominent representatives of phytochemicals with observed COX-2 inhibitory activity isolated from many plant species [15, 16]. In the context of this work, the focus was set on the investigation of triterpenoid- and steroidal-saponins, as they are the predominant representatives. Being composed of an aglycon (water-insoluble part) attached to hydrophilic glycoside moieties (water-soluble part), triterpenoid- and steroidal- saponins are extracted more efficiently depending on the aglycon structure and number of sugar moieties attached [17-19].

The determinations of the triterpenoid- and steroidal-saponin content in the individual extracts demonstrated a direct correlation with the solvent polarity used: the content of saponins increases with reduced polarity of the solvent (ethyl acetate > ethanol > methanol > water). These results indicated that the saponins in the leaves of *Waltheria indica* are predominantly present the form of aglycones or contain a small amount of sugar units. In addition to the observed influence of solvent polarity on the extraction rate of the saponins, an impact of extraction temperature was also established. The results demonstrated that at elevated temperatures during the extraction with ethanol, the content of the saponins in the extracts decreased. This observation suggests that saponins in *Waltheria indica* leaves dissolve easily in ethanol and are readily available in the plant matrix, but as the extraction temperature increases, further compounds are extracted from the plant material and the relative proportion of saponins in the final extract declines. The studies on the content of saponins further revealed that the content of triterpenoid-saponins was significantly higher than that of steroidal-saponins in all the extracts studied. The results make it apparent that saponins, along with phenols, belong to the substance classes that are most prominently represented in *Waltheria indica* leaves. These insights are of considerable relevance, as only qualitative studies on saponin content in *Waltheria indica* had been reported.

The comparison of the COX-2 inhibitory activity of the extract with the respective content of triterpenoid- and steroidal-saponins indicated a direct positive correlation. As outlined before, the extracts obtained exposed more prominent COX-2 inhibitory properties, depending on the solvent used, in the order ethyl acetate > ethanol > methanol > water, the identical sequence in which the saponins were preferentially

extracted from *Waltheria indica*. The observation that at elevated extraction temperatures the saponin content in the extracts decreased and simultaneously the COX-2 inhibitory activity of the extracts declined supported the assumption of a potential correlation. The investigation on the contribution of steroidal- vs. triterpenoid-saponins utilizing linear regression the results imply that the steroidal-saponins of *Waltheria indica* leave extracts are more potent COX-2 inhibitors than the triterpenoid-saponins.

At this stage it could not be excluded that other substances in *Waltheria indica*, which were preferentially extracted by non-polar solvents, might contribute to or be mainly responsible for the COX-2 inhibitory activity in the extracts. Nevertheless, the overall results indicated that the saponins contribute to the COX-2 inhibitory properties of the extracts to a certain but as yet unspecified extent. These findings are of importance as the anti-inflammatory properties of *Waltheria indica* extracts were linked to the phenolic compounds tiliroside or quercetin [4,5]. Although it has been reported that these phenolic compounds as pure substances possess COX-2 inhibitory properties, their concentration in the extracts were not taken into consideration. This presumably resulted in the false assumption that they are a major contributor to or are responsible for the observed COX-2 inhibitory activity of *Waltheria indica* extracts. In this context, it cannot be excluded that other anti-inflammatory properties of *Waltheria indica* are not the result of the substances reported so far, but of other unknown compounds.

The results demonstrate that although tiliroside inhibits the COX-2 activity in a concentration-dependent manner, the concentration of tiliroside in the plant was not sufficient to predominantly contribute to the activity of the extracts. Furthermore, the research revealed that the alkaloid levels were low and extracts with undetectable levels of alkaloids exhibited the highest activity. These findings led to the decision to not investigate them further as positive contributors to COX-2 inhibitory activity. For the attempt to design a predictive model for the COX-2 inhibitory effect of *Waltheria indica* extracts, it was indispensable to correctly identify the positive contributors to the observed COX-2 inhibitory activity.

Identification of compounds and their contribution to COX-2 inhibitory activity

The next step was to further identify and quantify molecules responsible for the COX-2 inhibitory activity of the extracts using an activity guided approach through extract fractionation by centrifugal partition chromatography (CPC). The simplification of the extract matrix was intended to enable a targeted identification of active fractions and subsequently the characterization of the compounds involved. Ideally, it should be verified whether and to what extent the COX-2 inhibitory effect correlates with the content of these substances in the extracts. In addition, the different CPC fractions produced were analyzed for their phytochemical composition in terms of total phenols, triterpenoid- and steroid-saponin content. In contrast to the complex extracts, the simplification in fractions based on the partition coefficient of the unknown compounds allow the separation of polar compounds e.g., phenolics from non-polar ones like saponins, or saponins from other non-polar compounds enabling a more precise conclusions to be drawn as to whether the individual phytochemicals contribute to the COX-2 inhibitory activity.

With the goal of obtaining fractions composed of different compounds of the *Waltheria indica* leaves the extract generated at 70 °C with ethanol was selected as reference sample. To realize the separation of

the extracts into distinct fractions, which in sum depict all components of the extract, a $2V_C$ Elution-Extrusion method was applied utilizing a CPC coupled with a UV detector. Using CPC allows for a high loading capacity of the system, with no need to completely dissolve the extract for separation, which allowed the injection of higher concentrations of extract. This ensured that with a reduced number of cycles and associated solvent consumption, fractions with sufficient matter were generated for further biological and analytical testing. During the investigations it became apparent that several compounds were not UV active, which included the saponins, as they do not possess a chromophore [20]. In particular, the fractionation of the non-polar part of the extract was partly realized on a visual basis, as the fractions showed color differences, with no detectable UV signal. Through a multi-stage process, involving the application of varying solvent compositions, the extract was successfully partitioned into five fractions. Three fractions represented the polar extract portion, whereby in one fraction tiliroside was enriched, and two represented the non-polar portion. A parallel analysis of the potential CPC fractions using UHPLC-CAD enabled this to be accomplished. The use of the CAD detector allowed the detection of UV-inactive compounds realizing a more appropriate selection of relevant fractions. The COX-2 activity of the CPC fractions show that all exhibit an inhibitory activity. However, the activity of the most active non-polar fraction was 2.8 times higher compared to the most active polar fraction. These findings further supported the assumption that the most active compounds with respect to COX-2 inhibitory activity in *Waltheria indica* belong to the class of non-polar molecules. Furthermore, it was observed that the polar fraction in which the tiliroside was enriched had a significantly higher activity compared to the other polar fractions. This result highlighted that tiliroside contributes to COX-2 activity when present in sufficient quantities in the plant and subsequently in the extract. Considering that non-polar fractions exhibited the highest activity, and thus supposedly containing the most potent compounds, these CPC fractions were selected to elucidate the structure of molecules, responsible for the COX-2 inhibitory activity.

Three substances in the fractions were successfully elucidated by means of high-resolution mass spectrometry (HPLC-MS/MS) based on the observed masses and matching with databases. Their verification was achieved by comparing the MS/MS fragmentation data and retention times with the respective reference standard. The molecules identified were the fatty acids (FA) alpha-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA), which were identified and reported for the first time in *Waltheria indica* leaf extracts. In the literature, all three compounds have been described as potential COX-2 inhibitors [21-23]. Investigations into the mechanism of action suggest that their inhibitory activity originates in competing with arachidonic acid as substrates for COX-2. The COX reaction produces prostaglandins by converting arachidonic acid. However, this enzyme is capable of oxidizing other unsaturated fatty acids such as ALA, LA or OA [24,25]. Structural determinations have revealed that, like AA, these FA bind in elongated L-shaped conformations within COX-2, thus blocking the active site for AA metabolism [26,27]. Based on the type of assay applied to determine COX-2 inhibitory properties, the reported findings vary considerably, with results indicating that ALA and LA are more effective than OA [21-23]. The investigations of ALA, LA and OA for their COX-2 inhibitory activities within the scope of this study demonstrated that the COX-2 inhibitory activity of ALA and LA increased in a dose dependent manner starting at a concentration of 0.9 µM, while OA showed a measurable activity starting

at 5 µM. These findings support the reported observations that ALA and LA exhibit superior COX-2 inhibitory activity over OA, which required a considerably higher concentration to exhibit an observable activity.

Having confirmed that the identified fatty acids possess COX-2 inhibitory activity, their content was quantified in the extracts with the objective to examine the influence of extraction parameters on their extraction from *Waltheria indica* leaves. The quantification of FA in extracts further enabled the verification whether their amount in the extracts was sufficient for the observed COX-2 inhibitory activity with the extracts.

The analyses of the extracts for their ALA, LA and OA content provided three key findings. First, it become evident that, regardless of the solvent or temperature used, when present in the extract ALA was the most abundant FA, followed by OA and LA. Secondly, the results highlighted the influence of the solvent polarity employed, with the FA content decreasing using more polar solvents, whereby neither of the examined FA were identified in the extract obtained with water as extractant. Here, the results also demonstrated that while the highest yields of the FA were found in the extract generated with ethyl acetate as the extracting agent, actually more FA were extracted per 1 gram of plant material with ethanol and methanol. The reason for the lower content of FA in these extracts can be attributed to the fact that they contain larger amounts of additional polar compounds, which could not be dissolved by ethyl acetate as extractant, resulting in a dilution of the fatty acids in the total extract. The third key finding related to the fact that the temperature during extraction made a significant difference on the content of FA in the extract. It was found that a higher extraction temperature resulted in a lower content of these FAs in the extract. In contrast, the total amount of ALA, LA and OA extracted from 1 g plant material increased at higher extraction temperatures. It can be stated that a higher temperature contributes to the disruption of the plant cell wall to enable the solvent to extract more lipids, while simultaneously extracting larger amounts of additional substances, resulting in a reduced content of fatty acids in the total extract. These overall observations were very similar to those made with saponins, which might be attributed to the similarly hydrophobic character of both compound classes.

Examining the COX-2 inhibitory properties of the extracts in relation to their FAs content revealed three major findings. First, the results indicate that COX-2 inhibitory activity of the extracts declines with the decrease of their FA concentration. Secondly, the content of OA in the extracts was found to be below the concentration at which the lowest COX-2 inhibitory activity was observed with the OA reference standard. Thirdly, it was not feasible to deduce the COX-2 inhibitory properties of the extracts based on the content of the individual FAs and the previously performed COX-2 assays with FAs reference standards. Using the COX-2 inhibition results of ALA and LA reference standards at different concentrations as a calibration curve and applying them to the extracts would lead to the expectation of either higher or lower COX-2 inhibitory activity, depending on the extract studied, than those actually determined.

With the aim to investigate the more accurate contribution of the FAs to the observed COX-2 inhibitory activity of the extracts, fatty acid mixtures (FAMs) containing ALA, LA and OA at concentrations and ratios identical to the corresponding extract were prepared and analyzed for their COX-2 inhibitory

activity. The results of the FAMs indicated a similar trend to the one obtained from the corresponding extracts, resulting in a decline of activity as the concentration of FAs decreases. Through the comparison of the COX-2 inhibitory activity of the FAM with the corresponding extract indicated that the contribution of the FAs to the COX-2 inhibitory activity of the extract ranged from a minimum of 31.3 % up to 41.6 %, whereby no conclusions on the influence of the extractant or temperature were apparent. These results are of relevance as they provide clear evidence that the identified fatty acids with their concentration in the extracts contribute substantially to COX-2 inhibition observed with the respective extract. From these findings emerged that preparation of *Waltheria indica* extracts with enhanced anti-inflammatory properties would be feasible by choosing extraction techniques and parameters that result in higher ALA or LA content in the extract. However, this experimental approach also highlighted the divergence between the increase in FA levels in the FAMs in comparison to their increase in COX-2 inhibitory activity. Exemplary in this context would be the comparison of the FAM containing the highest and lowest content of FAs. It was observed that while the mixture with the highest FA content contained factor 5.7 higher levels of ALA and factor 6.8 of LA, the enhancement of COX-2 inhibitory activity increased only by factor of 1.9. Whereas the investigations with ALA and LA reference standards demonstrated that in the concentration range as they were present in these FAMs, a linear relationship would be expected. Given that the inhibitory activity of ALA, LA and OA originates in competing with AA as substrate for COX-2, the additional interactions of ALA, LA and OA with one another are presumably more conducive to a higher rate of AA metabolism, in contrast to using pure compounds. These findings illustrate that although the research results obtained with pure compounds can be indicative, the actual outcomes may differ significantly due to unknown interactions of the compounds occurring in a complex matrix. At the same time, simplification of the plant matrix enabled targeted identification of compounds that contribute positively to the COX-2 inhibitory activity.

The contribution of the individual phytochemicals to COX-2 activity in a simplified matrix

Considering the unknown interactions of the compounds present in the extract, the phytochemical composition of the different CPC fractions generated was investigated. The CPC fractions obtained were used to determine the contribution of the individual phytochemicals in this simplified matrix to COX-2 inhibitory activity, which was fully apparent given the interferences in the complex extract matrix. The results demonstrated that the highest phenolic content was observed in the polar fractions, which were factor 10 higher to these in non-polar fractions, while no FAs were present in the polar fractions. By contrast, the polar fractions contained significantly lower levels of triterpenoid- and steroidal-saponins, whereby the saponins were found in every fraction. The presence of both triterpenoid- and steroidal-saponins in fractions independent of polarity indicates that both polar, presumably consisting of higher number of sugar moieties and carboxyl groups, and non-polar saponins are present in *Waltheria indica* leaves.

Recently, the triterpenoids (3β)-3-Hydroxy-lup-20(29)-en-28-oic acid (betulinic acid), β acetoxy-27-trans-caffeoyloxyolean-12-en-28-oicacid methyl ester and β-acetoxy-27-cis-caffeoyloxyolean-12-en-28-oic acid methyl ester have been reported to be present in aerial parts of *Waltheria indica* [14,28]. The HPLC-MS/MS analyses of the *Waltheria indica* leaf extracts in the framework of this study, however,

showed that these triterpenoids were not present or found. The reason is that the content and type of phytochemicals varies within plant parts (leaf, stem and root) [29]. The triterpenoids were identified in the aerial part including leaves and stems, whereas in this study the leaves without stem were investigated.

Detailed examination of the two non-polar fractions revealed that the triterpenoid- and steroidal-saponins as well as the FAs were enriched differently in these fractions. The fraction containing the most hydrophobic compounds contained a factor 1.7 lower amount of triterpenoid-saponins while containing factor 3.4 higher amount of steroidal-saponins. In addition, this fraction contained 12.5 times less ALA and 2.8 times less LA, whereas the OA content was comparable.

The separation of the different phytochemicals into distinct fractions provided a more detailed insight into the origin of the COX-2 inhibitory activity. When considering the total phenol content in the polar fractions and their COX-2 inhibition, a correlation was not evident as the fraction with highest phenol content demonstrated a lower COX-2 inhibitory activity compared to the fraction with lowest phenol content but highest COX-2 inhibitory activity. In the case of the triterpenoid- and steroidal saponin content, one could deduce that COX-2 inhibition increases with its increasing content in these fractions. Given the fact that the polar fractions do not contained FAs, the assumption was that their activity was predominantly originated by the saponins they contain. Based on these findings, phenols as present in *Waltheria indica* leaves were ruled out as positive contributors to the COX-2 inhibitory activity observed in the extracts.

In the case of the two non-polar fractions, the results revealed that although the fraction containing the most hydrophobic compounds contained lower amounts of triterpenoid-saponins, ALA and LA, the observed COX-2 inhibitory activity was factor 1.4 higher. An explanation for this observation might be the content of steroidal-saponins, which was 3.5 times higher in this fraction, suggesting that steroidal-saponins in *Waltheria Indica* leaves predominantly contribute to the COX-2 inhibitory activity in this case. These results further confirm the first observations with the extracts, which suggested that the steroidal-saponins play a prominent role in the COX-2 inhibitory activity. Considering the identical behavior of the FAs during the extractions with that of saponins and the similar effect on COX-2 inhibitory activity, these results further established that steroidal- as well as triterpenoid-saponins contribute positively to the COX-2 inhibitory activity of the extracts alongside ALA and LA.

Relationships between individual phytochemicals and their contribution to COX-2 activity

While the biological effects of new chemical compounds can often be predicted from its molecular structure using data of similar compounds, the prediction of the activity of complex mixtures such as plant extracts still remains a major challenge. Following the unequivocal identification of ALA, LA as well as steroidal- and triterpenoid-saponins as positive contributors to COX-2 inhibitory activity, they were used to for the attempt to develop a prediction model for the COX-2 inhibitory activity aimed to establish relationships between the individual phytochemicals and their contribution to the activity in the *Waltheria indica* leaf extracts. Tiliroside, OA and phenols were not taken into consideration since experimental

work indicated that they do not influence the COX-2 inhibitory activity at the concentration levels present in the extracts.

Two mathematical models (model A and B) were generated for the prediction of the COX-2 inhibitory activity of the *Waltheria indica* extracts. Model A was generated by considering as predictor variable for COX-2 inhibition activity the weighted sum of the concentrations of ALA, LA and the saponins as positive contributors in the individual extract. For each contributor the slope of the linear regression line between the concentration of the contributor and COX-2 inhibitory activity of the extracts served as the weighting factor. For ALA and LA a second-order polynomial relationship between their concentration and COX-2 inhibition activity was observed. Thus, a linearization was applied to both variables, in this case a square root transformation, to obtain the slope of the linear regression line. The obtained weighting factors for the individual concentrations were 0.27 for triterpenoid-saponins, 0.50 for steroidal saponins, 8.23 for square root of ALA and 10.03 for square root of LA. A linear regression line was then fitted between the weighted sum of contributors (x-variable) calculated using equation and COX-2 inhibition activity (y-variable), leading to the equation y = 0.257x + 20.911.

Model B was generated using partial least squares (PLS) regression, a linear multivariate regression useful to analyze data with many variables and few observations. The PLS regression considered as predictors (x-variables) the concentration of each positive contributor and the COX-2 inhibitory activity as response variable (y-variable). Similar to Model A, ALA and LA concentration values were first square root transformed to improve their linear relationship with COX-2 inhibitory activity. The obtained PLS model was represented by the linear equation y = 0.0693a + 0.1296b + 2.1269c + 2.5925d + 20.9035.

Both models were used to generate a linearity plot, i.e., the scatter plot showing the relationship between the observed versus predicted COX-2 inhibitory activity values for the extracts as shown in Figure 1. Applying the linear fit equation from model A or model B resulted in a similarly good fit with a root mean square error (RMSE) = 1.6% COX-2 inhibitory activity, thus demonstrating a high correspondence between predicted versus observed values.

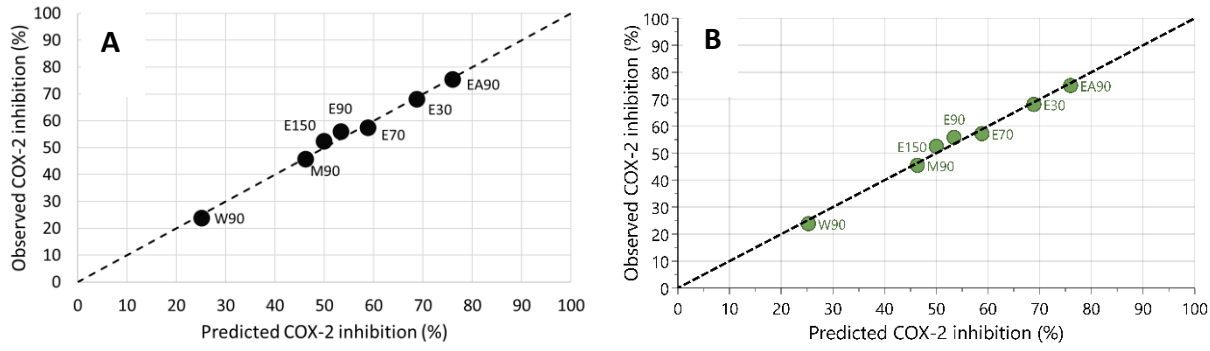


Figure 1: Graphs representing linearity plot of observed versus predicted COX-2 inhibition of Waltheria indica extracts obtained varying extraction temperatures and solvents. EA90, 90 °C ethyl acetate; E30-E70-E90-E150, 30-70-90-150 °C ethanol; M90, 90 °C methanol; W90, 90 °C water. **(A)** model A linearity plot (RMSE = 1.6 %) based on the obtained linear fit equation y=0.257x + 20.911 generated by the weighted sum of the contributors versus COX-2 inhibitory activity and **(B)** model B linearity plot (RMSE = 1.6 %) based on the generated linear fit equation y = 0.0693a + 0.1269b + 2.1269c + 2.5925d + 20.9035 by PLS regression. Dashed lines represent the line of identity (y=x). y=predicted COX-2 inhibition (%); x= weighted sum concentration of the contributors; a=triterpenoid-saponin concentration; b=steroidal saponin concentration; c=square root concentration of ALA; d=square root concentration of LA.

These results demonstrate that the approach of using weighted sum of the concentrations in model A led to similar results as the application of a multivariate regression method available using a commercial software carried out with model B. In both models, the data show that the coefficients of the contributors increase in the order LA > ALA > steroidal-saponins > triterpenoid-saponins indicating that their contribution to COX-2 inhibitory activity in *Waltheria indica* extracts follows this order. It is important to note that while these models provide good prediction results for the *Waltheria indica* extracts, they were not applicable to CPC fractions or to pure substances. As it is important not to extrapolate, the concentration of the phytochemicals in the test samples they should be within the concentration ranges of the extracts used for the models. While the individual fractions were simplified systems suitable for the identification of positive contributors to the COX-2 inhibitory activity, extracts are more complex with a greater number of putative interactions between the individual components with an influence on the potential biological activity. This was particularly evident from two observations. Firstly, examining the individual substances without any interactions with other compounds in the extracts, where ALA showed a stronger COX-inhibitory activity compared to LA. Secondly, in case of the CPC fractions where the concentration of several phytochemicals was higher compared to their concentration in extracts, the data suggested that the steroidal-saponins contribute the most. The results of the prediction models compared to the results of the simplified systems further highlighted that the interaction of the individual phytochemicals in the extracts play a dominant role and that the consideration of as many components as feasible was essential for a more accurate statement of where the observed activity originated from. In the future which lead to the discovery of additional substances that contribute positively to COX-2 inhibition and are present in sufficient concentration in the extracts, the mathematical models established in here should be reconfirmed and if required adopted accordingly.

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7. Outlook

The outcome of this thesis revealed that the fatty acids and saponins of *Waltheria indica* leaves predominantly contribute to their COX-2 inhibitory activity, which allowed the design of prediction models for the extract activity.

To understand which saponins are responsible for the activity, their structures need to be elucidated in detail. This is achieved by the combined use of nuclear magnetic resonance (NMR) and MS approaches, whereby NMR allows to assess the complete and definitive aglycone structure, including stereochemistry, the identification of sugars, the linkage positions, and sequence of the sugar chain. However, to elucidate the structure of completely unknown compounds by NMR, they need to be purified. Here, the CPC methods elaborated in this work are applied and needs to be further improved. Considering that the target compounds are of a hydrophobic character, the CPC should be operated in ascending mode, thus resulting in hydrophobic compounds being eluted first and therefore considerably shortening the operational time. In this process, the CPC needs to be coupled with mass spectrometry to enable saponin detection and allowing a precise fractionation. In addition, this approach can be applied to characterize additional molecules that are not be involved in COX-2 inhibitory activity but might be engaged in other biological functions.

With the aim of generating *Waltheria Indica* extracts with improved anti-inflammatory properties related to the inhibition of COX-2, extraction techniques should be applied where fatty acids and non-polar saponins would be primarily extracted. This can be assured by application of design of experiments (DOE) preferably including additionally to extractant polarity and temperature also extractant mixtures and further extraction methods as process variables. In this context, the utilization of supercritical CO_2 extraction is a promising alternative to the extraction with classic solvents. The physicochemical properties of supercritical CO_2 (higher diffusivity, lower viscosity, and lower surface tension than conventional solvents) facilitate mass transfer and allow an environmentally friendly extraction of primarily non-polar compounds.

For further verification of the prediction models developed for the COX-2 inhibitory effect of *Waltheria indica* leaf extracts, the model needs to be extended to other plants. The approach described in this project can be used to identify additional contributors to activity depending on the plant species and further broaden the model. The transfer of the approach presented in this project to the prediction of other observed biological endpoints with *Waltheria indica* extracts would be of great interest and will be considered in future studies.

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Publications

Peer reviewed articles

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Contribution to conferences

Talks:

Identification of active ingredients in plant extracts
(Marburg Conference on Nanopharmaceutics 2019, Marburg, Germany)

Separation of phytochemicals from crude plant extracts using CPC
(Marburg Conference on Nanopharmaceutics 2019, Marburg, Germany)

Poster:

Enrichment of bioactive phytochemicals from natural extracts by Centrifugal Partition Chromatography using polarity adjusted solvent regimes
(DPHG Jahrestagung 2019, Heidelberg, Germany)

Development of fractionation regimes for the separation of bioactive phytochemicals from crude natural extracts by Centrifugal Partition Chromatography
(DECHEMA 2019, Frankfurt, Germany)