Investigation of possible hazards and benefits of use of 'Ashkulebya' (Maerua subcordata (Gilg) DeWolf) as famine and/or potential functional food

Mebrahtom Gebrelibanos Hiben

Propositions

1. Wild edible plants may contribute to a healthy diet.

   (this thesis)

2. *In vitro* bioassays provide a useful first tier approach for studies on the potential health benefits and possible hazards of botanicals.

   (this thesis)

3. Preventing low grade chronic insults is a key approach to prevent chronic diseases.
4. The key to nature's therapy is trusting the body's inherent wisdom to heal itself.
5. Education brings the opportunity to make a difference.
6. A healthy environment is intrinsically linked to good human health.

Propositions belonging to the thesis entitled

Investigation of possible hazards and benefits of use of 'Ashkulebya' (*Maerua subcordata* (Gilg) DeWolf) as famine and/or potential functional food

Mebrahtom Gebrelibanos Hiben
Wageningen, 8 October 2019.

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Chapter 1

General introduction

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1. Plant domestication, bioactive compounds, and wild edible plants

Archaeological records indicate that during the Neolithic times and probably earlier, humans in various parts of the world began to confine and breed selected life forms. Modern agriculture is believed to have started in the Near East some 11,000 years ago with the domestication of figs, cereals, and legumes. At that time, early Neolithic farmers maintained a subsistence strategy, collecting wild plants for food and medicine while simultaneously domesticating early crops. This point in time marked the beginning of the divergence between medicinal plants and food plants [1,2].

Plants produce tens of thousands of unique natural products known as secondary metabolites or simply phytochemicals or bioactive compounds (BACs), which are responsible for the toxic or beneficial outcomes related to the use of plants [3-5]. Some of these BACs are thought to be responsible for the fact that some nutrient rich wild crops remain wild since they impart perceived undesirable food attributes such as poor palatability and, especially in the early days, they have been generally assumed to be irrelevant, sometimes even harmful, to human health. It is thought that the pharmacological side effects of food, often residing in poorly palatable compounds, were not likely preserved or even considered advantageous by our ancestors [2,3]. Hence, nutrient rich wild crops, containing such compounds, are thought to have remained wild; yet, edible especially during food shortage.

Moreover, even if centuries of plant domestication and post-domestication selection has vividly improved agriculture and food quality, over time, the phytochemical content of many plants has changed resulting in enhanced content of some BACs and diminished content of others. Some BACs that impart desirable attributes to food palatability, colour, flavour or other beneficial properties have been enhanced. Examples of such BACs may include flavonoids, carotenoids, and ingredients of spices, which are often responsible for the therapeutic qualities of familiar foods [2,6-9]. On the other hand, BACs imparting perceived undesirable attributes such as poor palatability or other physiological effects have often been diminished in concentration [2,10-12] resulting in the decline of these BACs from major crops to levels where average daily consumption cannot produce a measurable pharmacological effect [2]. In line with such opinions, it was shown that domesticated plant foods contain relatively small amounts of BACs whereas non-domesticated plants grown in the wild contain a higher level and a more varied phytochemical composition than cultivated plants [3,13]. BACs reported to have lower levels in cultivated crops, but higher levels in wild counterparts include cyanogenic glycosides, glucosinolates, glycoalkaloids, glycoproteins (lectins), isoflavones, sesquiterpenes, phenolic

compounds, and furanocoumarins [2,14-16]. Currently, the health benefits of these BACs received a heightened interest by the pharmaceutical and food domains and as a result, wild edible plants are being sought as a potential viable options to promote health and subsidize food security.

2. Wild edible plants as potential functional or famine foods

Nowadays, it is generally agreed that wild edible plants (WEPs) remain an ignored facet of food supply that could play a role in improving food security and promote human health as WEPs often have rich nutritional composition and high levels of health-promoting components [3,17-20]. WEPs, usually categorized as emergency or famine foods [21], provide a huge (often untapped) potential dietary biodiversity to support sustainable food systems and subsidise food security [22]. Today, more than one billion people in the world, mainly in developing countries, utilize wild vegetables in their daily diet. Alternatively, people of industrialized countries are rediscovering WEP species with the intend of promoting their domestication as promising new functional foods [23]. A functional food is defined as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" [3].

The concept of functional foods raises concerns about food security and the proper selection of an adequate diet, beyond the classical adequacy in energy, protein, essential fats, vitamins and minerals [3]. WEPs provide a rich source of functional food ingredients and hence, candidates of functional foods. In fact, natural products of plant origin have been used as prominent sources of prophylactic agents for the prevention and treatment of various diseases since ancient times. The fact that the link between food and health has been recognized since antiquity [24] is often exemplified by the ancient concept of Hippocrates ''let food be your medicine and medicine be your food'' that was reiterated in the 19th century and its validity verified by today's scientific knowledge [25-27].

Functional foods occupy the frontier between food and pharma. Nowadays, pharmaceutical and food domains share a similar interest to obtain and characterize new bioactive compounds from natural sources which can be used as new drugs, functional food ingredients or nutraceuticals - a term used to describe an intercept of nutrition and pharmaceuticals [24,28-30]. Nutraceuticals are food or food ingredients that have defined physiological effects. They do not easily fall into the legal categories of food or drug and often inhabit a grey area between the two (**Fig 1**) [31]. The majority of WEP species are perceived as having medicinal properties [32] and a plethora of bioactive compounds with medicinal and nutraceutical properties have been isolated from

these species [23], which may be the basis for the keen research interest on WEPs as a potential source of functional foods.

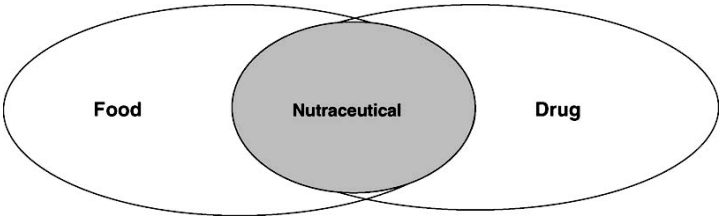

Figure 1. Nutraceuticals occupy a legal category between food and drug as described by Gulati and Ottaway, (2006) [31].

Equally important are the toxicological concerns about WEPs. Though WEPs are alternative sources of nutrients and nutraceuticals, they may be toxic due to their high content of some potentially toxic compounds implying that excessive and/or chronic consumption may cause some toxicity problems and hence initiate the need for a particular care with such species [23,32]. Indeed, the consumption of botanicals of potential concern for human health is cautioned by EFSA [32-34].

3. 'Ashkulebya' (*Maerua subcordata*): a wild food and medicinal plant

The present thesis focusses on *M. subcordata*, a wild food and medical plant of interest for use as famine and/or functional food. *M. subcordata* belongs to the caper family (Capparidaceae/ Capparaceae) [35,36], which is the tropical relative of the Cruciferae/Brassicaceae of temperate regions [35]. The caper family is a tropical and subtropical family that is well represented by woody species in Africa [36] containing a high number of wild edible [37] and medicinal [38] species used in Ethiopia. The genus *Maerua* is a woody genus with about 80 species distributed in the tropical and subtropical areas including 16 species present in the flora of Ethiopia and Eritrea. The genus is generally confined to shrubby savanna and semi-desert regions [36] and embraces species of trees or shrubs bearing edible, relatively large fleshy fruits [39]. The species *M. subcordata* (**Fig 2**) is a wild food and medicinal plant with a large tuber adapted to low-input agriculture and occurring in the dry parts of East Africa where its ethnobotanical claims are allied to three main applications: water purifying agent, food item, and herbal medicine that are detailed in **chapter 2** of the present thesis.

Figure 2. *Maerua subcordata* and its root tuber (left), and leaves and fruits (right)

4. Aim and objectives of the present thesis

The general aim of the present thesis intends to investigate ***'Ashkulebya'*** (*M. subcordata*) as a viable local source of potential functional food, to subsidise food security and promote heath, taking both possible benefits and risks into account. The study as such offers a proof of concept on how to evaluate possible dietary use of wild edible plants by providing scientific data that help to consider potential hazards and benefits, thereby supporting the rational use of botanicals. The objectives were:

1. To retrieve ethnobotanical background data on the wild (famine) food and medicinal plant, *Maerua subcordata* (Gilg) DeWolf, locally called 'Ashkulebya'
2. To employ reporter gene assays to identify potential health benefits of *M. subcordata* by a possible mechanism of induction of pathways of Nrf2/EpRE-mediated gene expression
3. To employ cell culture assays to identify potential health benefit of *M. subcordata* by a possible mechanism of induction of pathways of PPARγ- mediated gene expression and/or inhibition of pathways of NF-κB/iNOS mediated inflammations
4. To identify potential hazards associated with consumption of *M. subcordata* employing a battery of *in vitro* tests

To achieve these objectives some health-related cellular response pathways were chosen, which will be described below, focusing on background information on the endpoints used to identify potential health benefits and hazards of the botanical under study. In addition, classes of phytochemicals of interest in relation to these pathways are described in some more detail as well.

5. Selected phytochemicals

A large number of phytochemicals are found in plant-based foods like fruits, vegetables and grains, of which the intake has been associated with positive outcomes of overall health and

longevity [41,42]. The present section describes selected phytochemical groups that are of relevance for the present thesis. Glucosinolates and the alkaloids stachydrine and 3-hydroxystachydrine characterize the family Capparidaceae [42] to which *M. subcordata* belongs. Focus will be given to these and related compounds identified in this plant.

5.1. Glucosinolates

The order Capparales that includes Cruciferae, Capparidaceae, Resedaceae and Tovariaceae contain specific glucosinolates (GLs), and the enzyme myrosinase which, upon injury of the plant tissue, interacts with the GLs to produce volatile hydrolysis products, the isothiocyanates (ITCs) (**Fig 3**)[43,44]. GLs are sulfur- and nitrogen-containing plant secondary metabolites [45] that occur in plants that are widely used as food and medicine [46]. GLs and ITCs act as plant chemical defense against insects, herbivores and certain microbial pathogens. They have long been known for their allelopathic, bacteriocidal, fungicidal, and nematocidal properties. Lately, they attracted intense research interest because of their cancer chemoprotective attributes. Therefore, nowadays they represent a promising group of natural anti-infective and anti-cancer compounds [45,47-50].

Although these phytochemicals are attractive targets for the agricultural and pharmaceutical industries, the major focus of earlier research has been on the negative aspects of these compounds because of the prevalence of certain “antinutritional” or goitrogenic glucosinolates in the protein-rich defatted meal from widely grown oilseed crops and in some domesticated vegetable crops. There is, however, an opposite and positive side of this picture represented by the therapeutic and prophylactic properties of other “nutritional” or “functional” glucosinolates [47,51].

It is well known that consumption of Brassica vegetables has beneficial health effect on humans owing to their content of GLs and ITCs that are potential cancer preventing compounds. These compounds are also responsible for the sharp and bitter taste, and unique aroma characteristic of these vegetables, often causing rejection of this group of vegetables by consumers [52]. Some plants that are adapted to harsh dry climate provide nutritious food, but many of them contain GLs that are responsible for their bitter taste making them unpalatable. Such plants include certain species in the Capparidaceae such as *Boscia, Maerua, Cadaba,* and *Crateva* which form part of a long and deep food tradition in the Sahara region [53], implying that these plants may be potential functional foods and may contribute to food security in resource limited dry areas but also may promote health. Such a potential may also hold true for *M. subcordata*.

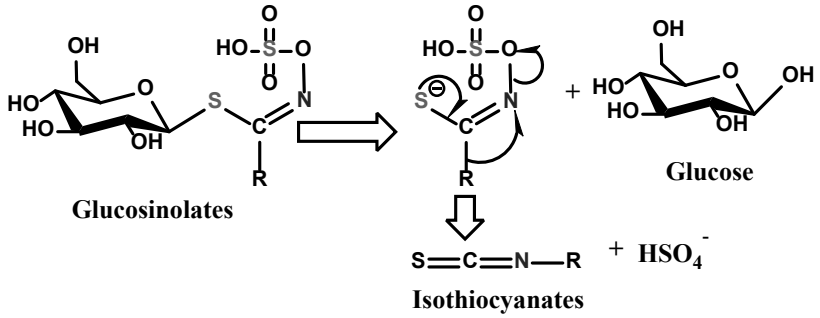

Figure 3. General structure of glucosinolates, which hydrolyse and undergo a rearrangement reaction to give isothiocyanates. Individual GLs/ITCs vary in their R group.

5.2. Betaines (quaternary ammonium compounds)

Betaines are quaternary ammonium compounds, ubiquitous in plants originating from amino and imino acids through specific biosynthetic pathways in response to abiotic stress that mainly function as osmoprotectants [54,55]. Historically, the term betaine was reserved for trimethyl-glycine after its discovery in the 19th century in the juice of sugar beets (*Beta vulgaris*). Betaine is called glycinebetaine to distinguish it from other betaines [56]. Some betaines are of particular interest in the human diet to maintain good health. Betaine itself is an important nutrient for the prevention of chronic diseases [57,58] and demonstrates anti-inflammatory effects [59,60]. Its dietary intake may contribute to overall health enhancement [58] and its use as a noble food was approved to be safe by EFSA [61]. Another vital betaine is stachydrine (prolinebetaine) which occurs at high levels in citrus juice. It was identified as a putative biomarker of citrus consumption for healthier eating patterns associated with a reduced risk of major chronic diseases [62]. Stachydrine and other related proline/hydroxyproline betaines were also identified in *Maerua spp*. including tetramethylammonium, 3-hydroxyl-1,1-dimethylpyrrolidiniurn, glycinebetaine (betaine), prolinebetaine (stachydrine) and its ethyl ester derivative, 3-hydroxyprolinebetaine (3-hydroxystachydrine), and choline and its feruloyl conjugate [63] (**Fig 4**). These constituents have also been identified in *M. subcordata* [63,64].

Like betaine, stachydrine was reported to exhibit potential prevention of chronic diseases due to its anti-cancer, anti-inflammatory, and anti-stress effects. Stachydrine was demonstrated to be cytotoxic to various cancer cell lines including prostate [65], breast [66], esophageal

squamous carcinoma [67]; and showed anti-tumour activity against induced gastric cancer in rats [68]. *In vitro* and *in vivo* studies showed that stachydrine inhibits inflammatory pathways, which was suggested as a likely mechanism for its cardioprotective and anti-hypertrophic [69-72], hepatoprotective [73], reduction of traumatic brain injury [74], reduction of cerebral impairment from ischemia re-perfusion [75], anti-endotoxin [76], and pilocytic astrocytoma suppression [77] effects. Also, it was reported to prevent endoplasmic reticulum stress-related apoptosis [78]; ameliorate high-glucose induced endothelial cell growth arrest and senescence [79]; protect against experimentally induced re-perfusion impairment of human umbilical vein endothelial cells [80]; reduce uterine bleeding of induced abortion in mice [81]; and act as antitussive by reducing citric acid-induced coughing [82]. Besides, the use of stachydrine in clinics, to promote blood circulation and dispel blood stasis, was reported [80].

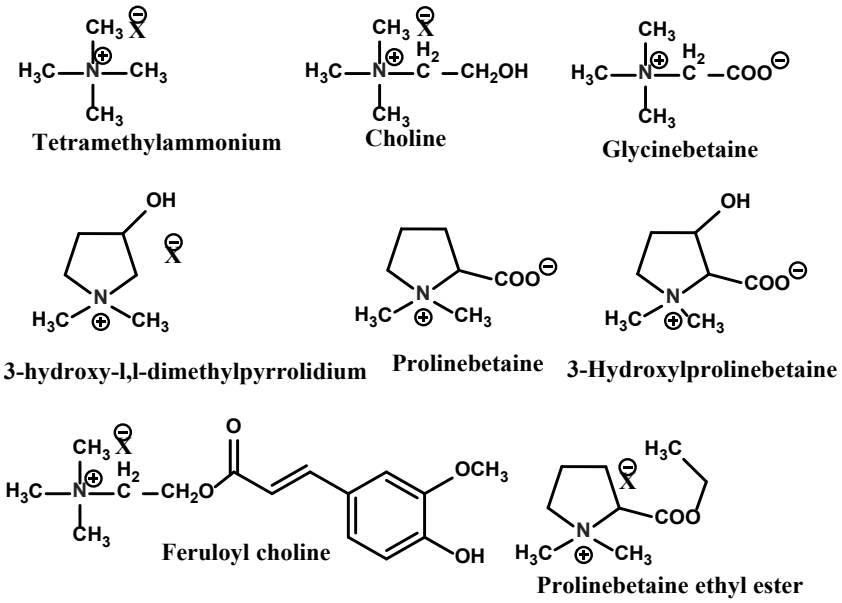

Figure 4. Structures of some betaines identified in *Maerua* spp of the caper family [63].

5.3. Agmatine and related guanidines

Biogenic amines are ubiquitous substances that play different roles in cellular metabolism, and mostly, polyamines act as bio-regulators of numerous cell functions [83]. In plants, polyamine metabolism changes in response to abiotic and biotic stress. Polyamines like agmatine occur in plant cells as free bases or as conjugated forms, commonly conjugated to cinnamic acids, and

usually accumulate in response to a variety of pathogens [84,85]. For example, cinnamoylagmatines such as p-coumaroylagmatine and feruloylagmatine tend to accumulate in response to fungal exposure and were shown to exhibit antifungal activity and they are direct precursors for the chemical defence of cereals [86,87]. Agmatine and its cinnamic acid conjugates were also identified in *Maerua spp*. such as *M. edulis* [85] and *M. subcordata* (**chapter 4** of the present thesis) along with other guanidine structures including galegine and various arginine biosynthesis products.

Agmatine and galegine showed blood glucose lowering effects and led to the development of the more potent modern-day biguanidine antidiabetics including metformin which is the first-line drug for type 2 diabetes [88-91]. In the 1920s, agmatine was explored as a potential nontoxic antidiabetic aminoguanidine analogue, until its derivatives, the modern day biguanidine anti-diabetics proved to be more potent [90]. Although it was discovered before a century, it recently received keen research interest, mainly in the field of clinical neuroscience aimed at testing its efficacy in the treatment of broad spectrum of neuropathologies [90,92-93]. Nowadays, several research findings endorse that agmatine modulates simultaneously multiple target sites, thus fitting the therapeutic profile of a 'magic shotgun' for complex clinical disorders of especially neuropathologies [90,93-95]. Yet, it is often reported to exert protective effects, including cardioprotection [96-98], gastroprotection [99], hepatoprotection [100], and nephroprotection [90].

Agmatine is biosynthesized from arginine by the enzyme arginine decarboxylase (ADC) (**Fig 5**) in bacteria, plants, and mammals [90,95,101,102]. It functions as key modulator of arginine metabolic pathways like nitric oxide production and polyamine metabolism thereby playing important roles in physiology and cellular repair mechanisms [90,97,103,104]. A proposed mechanism by which agmatine controls arginine metabolism including suppression of inducible nitric oxide synthase has been well described [90]. Yet, the endogenous level of agmatine in mammals is minimal, which limits its inherent benefits due to, at least, two reasons. First, agmatine biosynthesis by ADC competes with the principal arginine-dependent pathways that include the nitrogen metabolism (urea cycle), polyamine, nitric oxide, and protein synthesis [90]. Second, ADC activity in mammals is low, resulting in endogenous agmatine levels in mammalian tissues to be negligible, and hence the need for exogenous supply and a substantial quantity of agmatine to be of dietary origin [83].

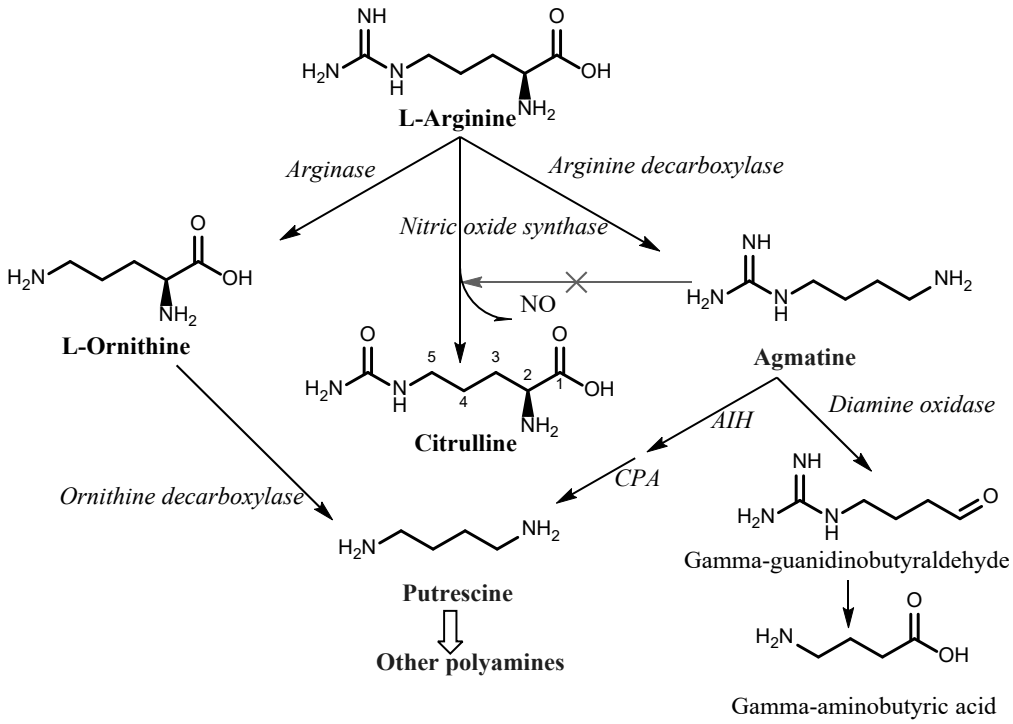

Figure 5. Biosynthetic and metabolic pathways of agmatine. Once produced from L-arginine, agmatine undergoes metabolism by agmatine iminohydrolase (AIH) and N-carbamoylputrescine amidohydrolase (CPA) leading to biosynthesis of other polyamines; it also acts as modulator of arginine metabolism *via* suppression of inducible nitric oxide synthase, among others.

6. Selected health related cellular response pathways

Every form of life is constantly exposed to a myriad of potentially lethal stimuli [105]. Thus, to survive under such environmental or intracellular stresses, eukaryotic cells have developed cellular defensive systems [105,106]. Likewise, humans are exposed, at intervals in lifetime, to several stressors, including drugs, various toxicants ingested via food, and phytochemicals in edible plants. To ensure survival in the face of such challenges, mammalian cells have evolved a variety of inducible genetic programs that enable them to adapt to the presence of harmful xenobiotics [107,108]. These programs entail upregulation of discrete batteries of genes for

drug-metabolizing enzymes, drug transporters, and various cytoprotective proteins that allow an increased rate of xenobiotic elimination from the body, restoration of normal homeostasis, and removal of damaged macromolecules [107].

These adaptive programs are evolutionary considered to have been genetically tuned to handle offensive phytochemicals. Plants have evolved a range of structural and chemical defenses to protect themselves from damage by animals. Contrariwise, animals counter-evolved pathways for the metabolism and adaptive cellular responses to toxic phytochemicals including numerous enzymes that degrade potentially noxious phytochemicals [109,110] but also other xenobiotics [108]. Such detoxifying processes typically involve three phases (I-III) although conventionally divided into two phases (I & II). Phase I reactions functionalize (add reactive and polar groups to) compounds by oxidation, reduction, and hydrolysis reactions. These reactions typically involve cytochrome P450 monooxygenases (CYPs). Phase II reactions support conjugation of phase I metabolites with endogenous ligands such as glutathione, glucuronic acid, and sulfate, catalyzed by glutathione S-transferases, UDP-glucuronosyl transferases, and sulfotransferases, respectively [108,110]. Phase III enzymes catalyze the ATP-dependent transport of xenobiotic conjugates of phase II, out of the cell, where they are then further metabolized and/or excreted [109].

Owing to these efficient mechanisms to detoxify and eliminate possibly toxic phytochemicals, cells are exposed only transiently to the phytochemicals [109]. Transient and/or sub-toxic exposure of phytochemicals may elicit mild adaptive cellular stress response pathways, thereby providing health benefits including protection against chronic diseases and delay of aging [109-111].

Phytochemicals can exert a protective effect by acting on multiple stress-response pathways [112] involving the activation of several transcription factors [109]. Among transcription factors that mediate xenobiotic adaptation, the aryl hydrocarbon receptor (AhR) and the nuclear factor-erythroid 2-related factor 2 (Nrf2) have been widely studied [107]. Adaptive stress response mechanisms that can be triggered by phytochemicals and may mediate health-promoting actions include the (i) Nrf2, (ii) nuclear factor-kB (NF-κB), and (iii) peroxisome proliferator-activated receptors (PPARs) [109]. Below are briefly highlighted selected health related adaptive cellular response pathways, which are of relevance to the present thesis.

6.1. AhR/XRE pathway

The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor that acts as a pivotal chemical sensor, which transduces extrinsic and intrinsic signals into cellular responses.

Originally thought to be involved in not only drug metabolism but also carcinogenic and toxicological responses against environmental contaminants like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), recent studies demonstrate that the AhR plays multiple intrinsic roles in host defense and homeostasis as well, including immunity, stem cell maintenance, and cell differentiation, upon binding with an increasing number of newly defined dietary, cellular, and microbe-derived ligands [113-114]. Unliganded AhR is located in the cytoplasm forming a complex with several chaperones among which are two heat shock protein 90 (HSP90) molecules, at least one immunophilin-like protein hepatitis B virus X-associated protein 2 (XAP2), and a co-chaperone p23. Binding of a ligand leads to a conformational change, thereby allowing nuclear translocation of the AhR complex. In the nucleus, the AhR dissociates from the complex and heterodimerizes with AhR nuclear translocator (ARNT). The AhR-ARNT heterodimer binds to AhR responsive elements (AHREs, also known as dioxin responsive elements (DRE) or xenobiotic responsive elements (XRE)) in the promoter of target genes that encode several phase I and phase II metabolizing enzymes (**Fig. 6**) but also of several other genes [114-117].

This pathway is referred to as the canonical pathway, which is inherently intended to mediate the detoxification of AhR ligands [114,115] although it may also lead to the conversion of some ligands into a more toxic metabolite. AhR activation by this pathway results in up-regulation of the expression of the so-called AhR gene battery that includes hundreds of genes involved in a wide variety of cellular processes, such as apoptosis, regulation of the cell cycle, and biotransformation of xenobiotics by phase I and II xenobiotic metabolizing enzymes, such as cytochrome P450s, glutathione-S-transferases, NADPH/quinone oxidoreductase, and UDP glucuronosyl transferases [114-116].

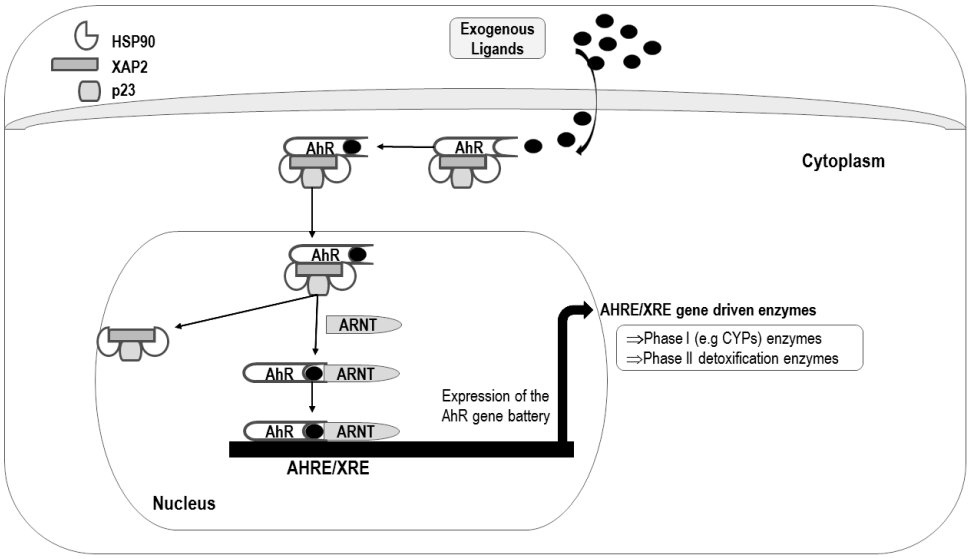

Figure 6. Canonical pathway of ligand induced AhR activation. A cytosolic AhR is complexed with two molecules of Hsp90, a XAP2, and a co-chaperone p23; upon ligand binding, the AhR complex is translocated into the nucleus where the AhR is dissociated from the complex to heterodimerize with ARNT. The AhR-ARNT heterodimer then binds to xenobiotic-responsive elements (XREs) in the promoter of genes that encode, amongst others, several phase I and phase II metabolizing enzymes.

Despite several essential roles of the AhR in cellular adaptive responses, it is also involved in various carcinogenic and toxicological responses [118]. What factors distinguish a beneficial response from a toxic response remains to be uncovered. Two suggested possibilities include (i) differential AhR activation by different ligands and (ii) timing of AhR activation. The fact that there exist diverse AhR ligands varying from synthetic ligands (often triggering toxic responses) to ligands of natural origins (often triggering health benefits) and endogenous ligands (having physiological functions) has led to the suggestion that the different ligands of the AhR may activate the receptor in a different way [117]. Recent reports show the activation, by TCDD, of some AhR dependent non-canonical pathways that may converge to regulate pathophysiological processes such as inflammation [117] implying that the AhR may be a convergence point for several signaling cascades, possibly involved in the diverse adverse effects caused by TCDD, a well-known AhR ligand with extremely high affinity and persistent binding [113]. Another opinion links the outcome of AhR activation with timing (i.e. the

duration of AhR signalling, rather than the nature of the receptor agonist, determines the biological outcomes after receptor activation) [118]. It has been shown that although 6-formylindolo[3,2-b]carbazole (FICZ), an endogenous AhR ligand, has greater binding affinity than TCDD and shares similar molecular targets with TCDD, transient versus sustained AhR activation by FICZ and TCDD caused differential transcriptomic responses [119]. Therefore, transient AhR activation by molecules like FICZ may be essential for the putative role of the AhR in cell homeostasis [120] while sustained AhR activation by molecules like TCDD may culminate in toxic responses [118].

6.2. Nrf2/ARE (EpRE) pathway

The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor is at the center of the day-to-day biological response to oxidative and electrophilic stress, which regulates the transcription of many antioxidant genes that preserve cellular homeostasis and detoxification genes that process and eliminate electrophilic carcinogens and toxins before they can cause damage [121]. Under conditions of redox homeostasis, Nrf2 is sequestered in the cytoplasm and targeted for ubiquitination and proteasomal degradation by Kelch-like ECH-associated protein 1 (Keap1). Nrf2 activating agents trigger Nrf2-mediated gene expression either by directly modifying the cysteine (Cys) residues on Keap1 to disrupt the Nrf2-Keap1 complex, or by provoking some kinase signaling pathways to phosphorylate the Nrf2-Keap1 complex and facilitate the release of Nrf2 [105]. Once freed from Keap1, Nrf2 translocates into the nucleus where it complexes with Small Maf proteins and subsequently binds to the antioxidant/ electrophile-responsive element (ARE/EpRE) in the regulatory domains of Nrf2 target genes (**Fig 7**) [105,110]. Genes induced by Nrf2 encode proteins of two major categories: antioxidant enzymes and phase 2 detoxification enzymes [110]. The Nrf2 pathway plays a major role in health resilience and can be made more robust and responsive by certain dietary phytochemicals [121]. The dark side of Nrf2 is that genes encoding Nrf2 and Keap1 are often mutated and constitutively activated in tumours, and hence equip cancer cells with a better defence against oxidative stress, enhancing their survival and resistance to chemotherapies and radiotherapies [114,122]. In such a situation, Nrf2 inhibitors might be helpful as adjuvants to chemotherapies.

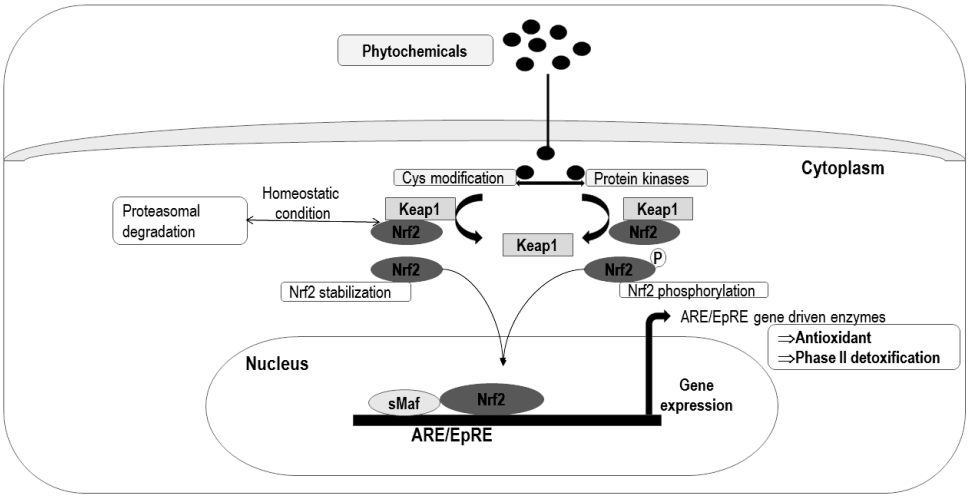

Figure 7. Nrf2-activating compounds including some phytochemicals cause Cys modifications of Keap 1 or trigger certain protein kinases that phosphorylate Nrf2, both resulting in the release of Nrf2 from Keap1. Freed Nrf2 then moves into the nucleus where it forms a complex with small Maf proteins and this complex binds to the ARE/EpRE, thereby enhancing transcription of the target genes.

6.3. PPAR/PPPE pathway

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors belonging to the nuclear receptor superfamily. Three subtypes of PPARs (PPARα, PPARγ, and PPARβ/δ) play a major regulatory role in energy homeostasis and metabolic function [123,124]. In the nucleus, PPARs exist as heterodimers with retinoid X receptor-α (RXR) bound to DNA with corepressor molecules. Upon ligand activation, PPARs undergo conformational changes that facilitate the dissociation of corepressor molecules and invoke recruitment of transcription cofactors including coactivators and coactivator-associated proteins [125,126]. The liganded PPAR/RXR heterodimers bind to specific DNA sequence elements, termed peroxisome proliferator responsive elements (PPREs), and regulate expression of target genes [123,127,128] (**Fig 8**) involved in energy homeostasis and metabolic function [124]. PPAR ligand binding leads to interactions with co-activators and/or co-repressors to induce or inhibit their functions. PPAR functions are mainly regulated through ligand binding, but also by some posttranslational modifications such as phosphorylation, SUMOylation, ubiquitination, and acetylation which are found at numerous modification sites [129].

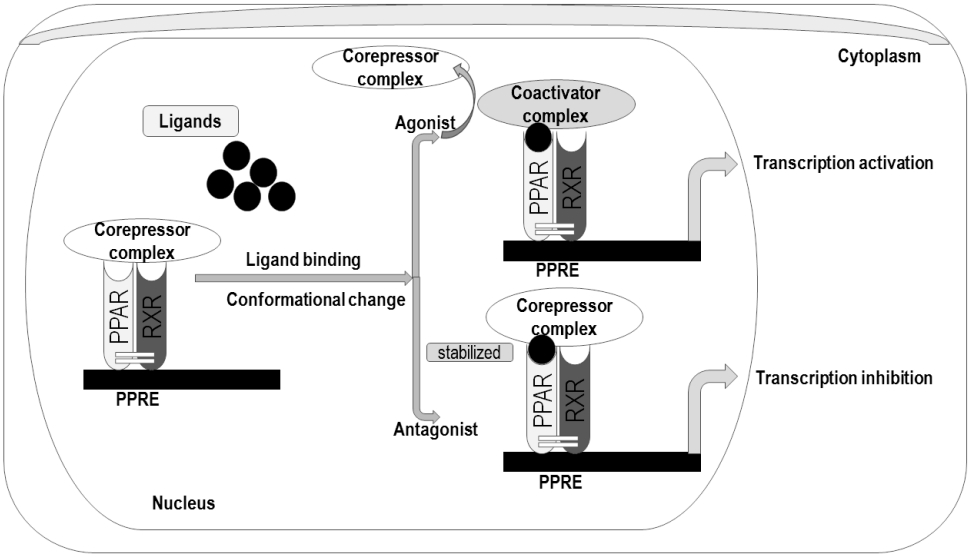

Figure 8. PPAR/RXR heterodimers bind to DNA responsive elements (PPRE) in the regulatory regions of target genes. Liganded PPARs undergo conformational changes resulting in the recruitment of coactivator proteins (if the ligand is an agonist) leading to transcriptional activation or corepressors remain stabilized (if the ligand is an antagonist) leading to transcriptional repression.

Many natural dietary compounds, especially phytochemicals, are PPAR activators and ligand binding to PPARs has been proven to control several pathological conditions linked with obesity, aging-related diseases, inflammation, immune disorder, cell cycle regulation, and cancer [125]. The activation of PPARα reduces triglyceride levels and is involved in regulation of energy homeostasis; PPARγ activation causes insulin sensitization and enhances glucose metabolism, whereas activation of PPARβ/δ enhances fatty acid metabolism. Thus, the PPAR family of nuclear receptors plays a major regulatory role in energy homeostasis and metabolic function [124].

More importantly, the majority of identified natural ligands are reported to be selective PPARγ modulators [130]. As PPARγ activation by agonists leads to adipogenesis, exposure of people to prolonged chronic levels of PPARγ ligands, like in the westernised lifestyle, has been linked with obesity. Likewise, potent PPARγ agonists have the drawback of risk of obesity leading to the idea that the most effective strategy for resetting the balance of this gene is *via* its

modulation rather than full activation, with the goal to improve glucose homeostasis while preventing adipogenesis [131,132]. With this regard, PPARγ agonist natural products and plant extracts were shown to act as selective modulators and hence described as good potential candidates to be explored for their therapeutic effectiveness as dietary supplements to counteract the metabolic syndrome and type 2 diabetes [130].

6.4. ER signaling pathway

Estrogen receptors (ERs) are ligand-regulated transcription factors that belong to the nuclear receptor superfamily [133,134]. ERs function by three general modes of action: DNA-binding *via* estrogen responsive elements (EREs) (designated nuclear ERE signaling) (**Fig 9**); nuclear signaling *via* protein-protein interactions to other transcription factors (nuclear non-ERE signaling); and extra-nuclear signaling (membrane-bound functions of ERs) [135]. ERs (ERα and ERβ) are involved in the physiological development and metabolism in humans. They function as signal transducer and transcription factors to regulate the expression of target genes, thereby playing vital roles in the treatment of cancer and metabolic diseases [133,134]. ERα is essential for the maturation and function of the reproductive system, bone development, cognitive system development, and metabolism. While ERβ contributes less to these aspects than ERα, it does play an important role in prevention of disease. Due to the important functions of ERs in human development and health, searching for potent ER modulators remains an active area of investigation [134].

Abnormal ER signaling leads to development of a variety of diseases, such as cancer, metabolic and cardiovascular disease, neurodegeneration, inflammation, and osteoporosis. Many adverse effects of estrogens are involved in the activation of ERα signaling. In general, ERα expression increases at early stages of cancer, and acts as a tumor promoter, while ERβ levels are reduced during carcinogenesis and cancer progression, and act as a tumor suppressor [133,134]. Besides, various industrial chemicals can disrupt physiological responses to the ER in healthy people, and thus act as endocrine disruptors. Interestingly, many natural products in plants, so called phytoestrogens, are structural and functional analogues to endogenous estrogens and act as ER transcriptional activators and modulators [134,136].

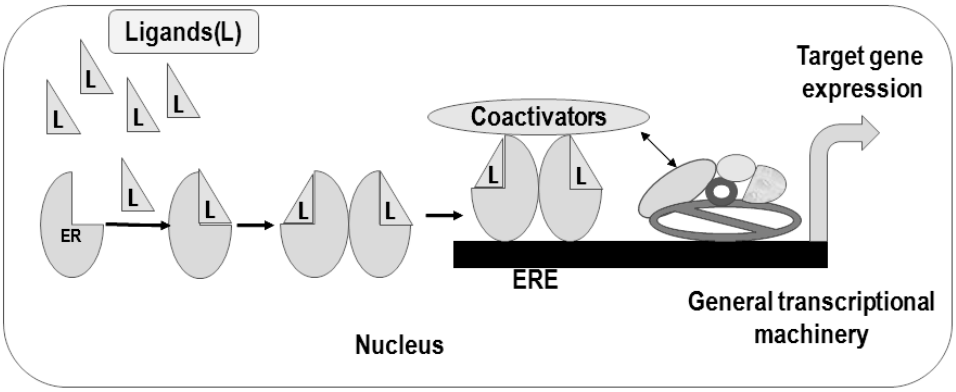

Figure 9. Ligand (L) binding to estrogen receptors (ERs) leads to dimerization and subsequent increased affinity for the estrogen-responsive element (ERE), and coactivators serve as a bridge between the receptor(s) and the general transcription machinery.

6.5. NF-κB/κB pathway

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is an important transcription factor that regulates the expression of genes involved in a range of biologic processes including innate and adaptive immunity, inflammation, cellular stress responses, proliferation, and cell survival [110,121]. The NF-κB (depicted as p65 and p50 heterodimer) is a protein complex ubiquitously expressed in almost all animal cell types, which under normal conditions is sequestered in the cytosol by NF-κB inhibitor protein (IκB) [110,121,137]. In response to a variety of stimuli including inflammatory cytokines, bacterial or viral antigens, and oxidative stress, IκB is phosphorylated by the IκB kinase (IκK) complex and ubiquitinated for its proteasomal degradation, releasing NF-κB to translocate into the nucleus [105,110,121,137] where it regulates gene expression by binding to specific DNA elements, collectively known as κB sites, that are located within the promoters/enhancers of target genes. Through sensing of central base pair differences within κB sites, NF-κB dimers modulate biological programs by activating, repressing, and altering the expression of effector genes including genes for inflammatory mediators, such as cellular cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [138] (**Fig 10**).

The NF-κB regulates cellular immune responses to infection and higher order oxidative stress [110,121,137]. Phytochemicals act at different targets in the NF-κB pathway to exert their immunomodulation and chemopreventive effects. Chemopreventive agents can suppress NF-

κB activation by blocking IκK activation, inhibition of IκB phosphorylation, and the direct phosphorylation of the p65 subunit [105]. COX-2 and iNOS are important enzymes that mediate inflammatory processes. Improper up-regulation of COX-2 and/or iNOS has been associated with pathology of inflammatory disorders as well as certain types of human cancers. Several chemopreventive phytochemicals have been shown to inhibit COX-2 and iNOS expression by inhibiting NF-κB activation [139].

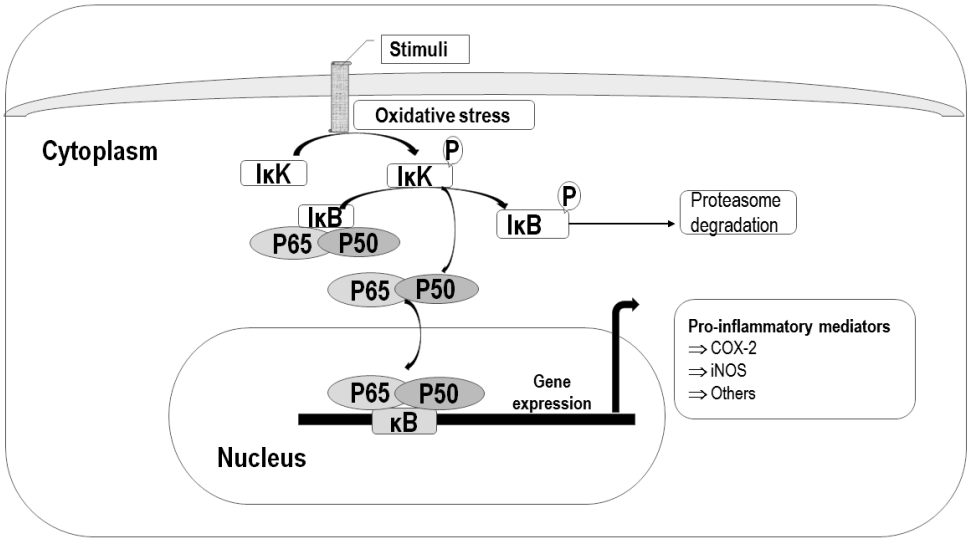

Figure 10. Activation of certain receptors (e.g. toll-like receptor 4) induces a signalling pathway leading to the activation of the transcription factor NF-κB: this involves phosphorylation of IκB kinase (IκK), then phosphorylation of IκB followed by its dissociation from the inactive cytosolic NF-κB trimer, and then its degradation. The active NF-κB dimer then translocates to the nucleus and initiates transcription of genes for inflammatory mediators, such as cellular COX-2 and iNOS.

7. Bioassays

Studies on botanicals and their active ingredients pose great challenges and complications due to, among others, costs, time, and animal usage plus the multicomponent nature of the phytoconstituents that often show variability in composition. This quests an integrative testing strategy using a panel of available alternative tests including optimal *in silico* and in vitro filters tailored to identify biologically relevant endpoints that can be used as an initial data for the

assessment of the potential health benefits or possible risks of botanical products [140] as well as industrial chemicals. Among several alternative tests, reporter gene assays and tests that use zebrafish as a model organism are emerging as leading and versatile test models. Moreover, other commonly used alternative tests include the embryonic stem cell test for developmental toxicity studies as well as the Ames test and *in silico* predictions for initial genotoxicity screening.

7.1. Reporter gene assays

Dietary and medicinal plants represent precious resources of bioactive compounds, which have several health implications [141,142]. A wide spectrum of bioassays can be employed for the detection of bioactivity in extracts, fractions, and purified compounds of herbal origin. Amongst the different types of bioassays, reporter gene assays are highly versatile and reliable [142] and can be applied for screening these natural products of plant origin for their capacity to induce gene expression through a specific health-related transcription factor [141]. Gene expression studies can be facilitated by using a reporter gene assay. The promoter region of the gene of interest can be cloned in front of a reporter gene enabling measurement of the reporter gene expression as a measure of the expression of the specific gene of interest [143] (**Fig 11**). The cloned reporter gene construct is often made to be part of a promoter (enhancer) fragment from a gene of interest under transcriptional control of a response element for the transcription factor of interest [144,145]. Binding of the transcription factor to the promoter results in the induction of the reporter gene (and subsequent reporter protein) expression in response to signaling pathway activation by external stimuli, including phytochemicals and drugs [145,146]. Cell-based reporter gene assays can be designed to measure the influence of a compound on a cellular process or pathway *via* the modulation of transcription and expression levels of the reporter-gene. Among the reporter gene assays, luciferase assays are often the method of choice for high-throughput screening for a number of reasons, mainly due to their bright signals and nearly non-existent background luminescence as no excitation light is required to generate a signal [147,148]. Genes encoding luciferase enzymes have been cloned from several species, including firefly [145]. By coupling a response element controlled by the signaling event of interest to luciferase, reporter gene assays can quantify an intracellular response to a biological event [149]. Firefly luciferase catalyses the oxidation of the substrate (luciferin) [145] producing luminescent light (**Fig 11**) that can be measured using a luminometer, and the produced signal is proportional to the evoked effect. Reporter gene assays used in the present

thesis included EpRE LUX, PPARγ CALUX, AhR CALUX, and ERα CALUX luciferase reporter gene assays as used in **Chapters 3-5**.

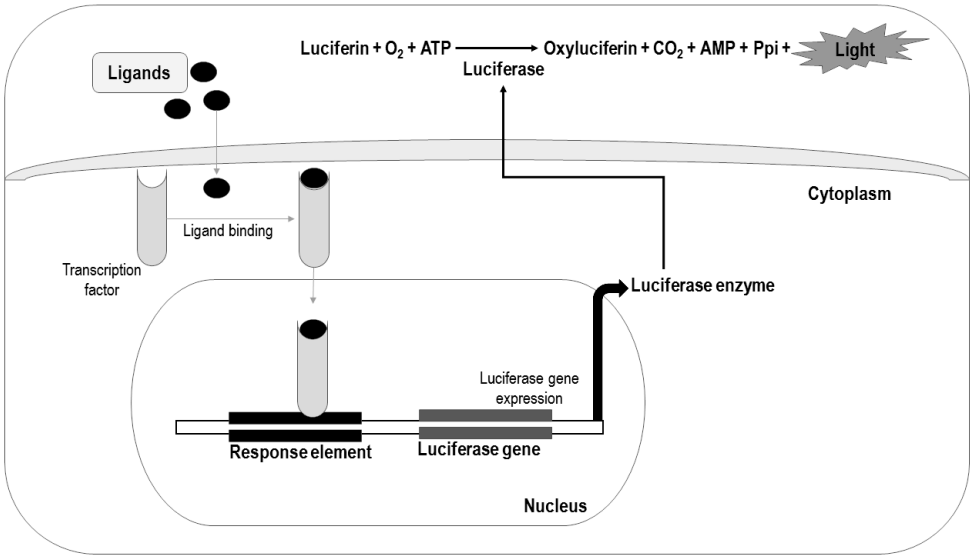

Figure 11. General overview of luciferase-based reporter gene assays. When stimuli elicit a cell to generate a response, a reporter gene (luciferase) is transcribed and translated into an enzyme, which in the presence of substrate (luciferin), produces luminescent light that can be measured using a luminometer.

7.2. The embryonic stem cell test (EST) for developmental toxicity testing

The EST is a procedure that utilizes comparison of cytotoxicity and inhibition of differentiation of ES-D3 stem cells into beating cardiomyocytes to predict embryotoxicity of compounds or botanical extracts tested [140,150]. Because of its use of inhibition of differentiation as an endpoint, the EST is extremely versatile in the range of complications it can test for [150]. In this thesis, the inhibition of differentiation of ES-D3 cells into beating cardiomyocytes has been employed as a bioassay to detect in vitro developmental toxicity.

7.3. Ames test and *in silico* prediction of genotoxicity

The Ames test is a widely accepted short-term bacterial (*Salmonella typhimurium*) assay for identifying substances that can produce genetic damage that leads to gene mutations [151]. A guidance document has been drafted by the European Medicines Agency on the assessment of

genotoxcicity of herbal preparations. The first stage of the guidance utilizes the Ames test and suggests no further genotoxicity testing in case of a negative result [152]. In recent years, the *in silico* prediction of genotoxicity has made considerable progress. This was made possible by the growing amount of reliable Ames screening data, the attempts to understand the activity pathways, and the subsequent development of computer-based prediction systems. The *in silico* prediction of genotoxicity provides an established and accepted method that defines the first step in the assessment of DNA reactive substances including plant chemicals [140,153].

7.4. The Zebrafish model

The zebrafish (*Danio rerio*) is a versatile and prominent model organism that has been used for decades in biomedical research to study a wide range of biological endpoints for therapeutic and/or toxicological applications [154,155] and in modelling of human diseases [156,157]. It is one of the best leading models to study developmental biology, toxicology, drug discovery, cancer, and molecular genetics [154,157]. Particularly the use of embryos up to 120 hours post fertilization (hpf) is getting increasing attention, since they are considered as replacement method for animal experiments [158]. As per the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, the earliest life-stages of animals are not defined as protected and, therefore, do not fall into the regulatory frameworks dealing with animal experimentation. Independent feeding is considered as the stage from which free-living larvae are subject to regulations [158,159]. Zebrafish larvae can be regarded as independently feeding from 120 hpf and experiments with zebrafish should thus be subject to regulations for animal experiments from 120 hpf [158]. Zebrafish embryos allow the analysis of multiple endpoints ranging from acute and developmental toxicity determination to complex functional genetic and physiological analysis [158].

8. Outline of the thesis

The main aim of the present thesis was to provide scientific support towards a rational use of Ashkulebya' (*Maerua subcordata* (Gilg) DeWolf) as a functional or (famine) food. To this end various lines of research were initiated providing evidences enabling evaluation of potential hazards and benefits. **Chapter 1** describes relevant background literature to show the potential of wild edible plants as possible functional foods and the influence of domestication on the content of ingredients responsible for the perceived health impact of plant based foods and medicines. Moreover, background information on the endpoints used to identify potential health benefits and hazards of a botanical indicated in the different topics of this thesis are described.

In **Chapter 2,** ethnobotanical data on 'Ashkulebya', the study plant in the present thesis, are retrieved and documented to support and design further experimental studies. In order to relate some of the biologically relevant endpoints for which 'Ashkulebya' extracts were tested in the bioassays used in **Chapters 3-5**, to possibly responsible active ingredients in these extracts, liquid chromatography coupled to multistage mass spectroscopy followed by 'MS Annotation based on in silico Generated Metabolites' (MAGMa) software structural annotation was applied to identify candidate constituents in extracts of the plant. **Chapter 3** describes the potential health benefits of *M. subcordata* (fruit, leaf, root, and seed) extracts and selected candidate constituents by assessing electrophile-responsive element (EpRE)-mediated gene expression induction using EpRE-LUX cell lines. **Chapter 4** describes the potential health benefit of *M. subcordata* (fruit, leaf, root, and seed) extracts and selected candidate constituents in relation to metabolic syndrome and inflammation by evaluating their potential induction of peroxisome proliferator activated receptor γ (PPARγ) mediated gene expression in U2OS-PPARγ2 cells and the inhibition of gram-negative bacterial lipopolysaccharide induced nitric oxide production in RAW264.7 macrophages. **Chapter 5** covers an *in silico* toxicity evaluation as well as different *in vitro* tests including the Ames test, the estrogen receptor alpha (ERα) CALUX reporter gene assay, the aryl hydrocarbon receptor (AhR) CALUX reporter gene assay, the embryonic stem cell test (EST), and the zebrafish embryotoxicity test (ZET) applied to identify potential health hazards of *M. subcordata* (fruit, leaf, root, and seed) extracts and selected candidate constituents. **Chapter 6** recaps results of the previous chapters and provides overall discussions and future perspectives.

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Chapter 2

Ethnomedicine and Ethnobotany of *Maerua subcordata* (Gilg) DeWolf

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Abstract

Wild edible plants are valuable resources for improving food and nutritional security. Besides, they may provide important health benefits since the health-promoting components of plant based foods usually exist at higher levels in wild plants. As a result, they are being sought as under-exploited potential sources of a health-promoting diet or a possible strategy to develop novel foods. In such exploration, ethnobotanical and ethnomedicinal data offer a fundamental step. The present study provides ethnomedicinal data on *Maerua subcordata* (Gilg) DeWolf (Capparidaceae). The ethnomedicinal data was collected from the Kunama ethnics of northern Ethiopia *via* focus group discussion and oral interview. Supporting ethnobotanical data from relevant literature was also compiled and systematically reviewed. The results show that *M. subcordata* tuber is used by the Kunamas to manage malaria, malaria symptoms (fever, pain, gastrointestinal disorders) and seasonal cough while leaves are used for wound healing. In east Africa, its triple potential use as water purifying agent, food item, and herbal medicine was specified. As a herbal medicine, the tuber is used to manage a wide range of disorders including pain, infections, wounds, diabetes, blood pressure, and loss of appetite. Its use as laxative and abortifacient was also indicated. Leaves are used to treat wounds, ophthalmic and respiratory problems. As a food item, fruits are eaten during times of both food scarcity and food abundance while the tuber is used as a famine food. In conclusion, *M. subcordata* represents a wild food and medicinal plant, which may be developed into a functional food or alternative medicine.

Key words: *ethnobotany, ethnomedicine, Kunamas*, *Maerua subcordata, wild edible plants*

1. Introduction

Plants are irreplaceable food resources for humans and virtually all human foods are plants or organisms that eat plants [1]. Ethnobotany is the study of the interrelations of man and plants [2] while ethnomedicine is especially concerned with the cultural interpretations of health and disease, which also addresses the traditional health care seeking process and healing practices [3,4]. In traditional medicines, mainly wild gathered food plants are often reported in different ethnic societies of local and popular traditions to have pharmacologic activities and are often associated with beneficial effects [2,5]. Wild food plants refers to all plant resources outside of agricultural areas that are harvested or collected from the wild for the purpose of human consumption [2].

Wild edible plants (WEPs) represent a category of foods that are virtually unexplored, usually consumed in times of famine and scarcity, have neglected role as foodstuffs for regular intake during times of sufficiency, and often categorized as emergency or famine foods [6-8]. WEPs, though underutilized, are still consumed by different societies and are gaining keen scientific interest owing to their nutritional and medicinal values that may broaden the diversity of the human diet and the connection between food and health [8-10]. WEPs remain an ignored facet of food supply, which may improve food security and promote health since many of them possess rich nutritional composition and higher levels of health-promoting components [6,7, 11-13]. Thus, it was suggested that some of these "neglected" species, sometimes considered as weeds in extensive major crop cultivation, may potentially become "new functional crops" [10].

WEPs are an integral part of the cultural and genetic heritage of different regions of the world [8]. Chiefly, indigenous dwellers in the rain forests of Africa and South America utilize WEPs as a food source, who gather and consume WEPs as snacks and at times of food scarcity. Likewise, the rural populations in Ethiopia have a rich knowledge of WEPs and consumption of such plants is still an integral part of the diverse cultures in the country [7]. Many WEPs in Africa are highly adapted to harsh growing conditions and are available when other sources of food fail or are out of season. They are often rich in macro and micro nutrients and health promoting components. They may provide vital option to promote food security and wellness owing to their availability and affordability, higher nutritional values, and health-promoting properties [14,15]. However, current research and agricultural development agenda, especially in Africa, still appear to focus on the popular and commonly used food crops, ignoring these

important WEPs [14]. Thus, despite their high biodiversity, rural populations in developing countries often face food insecurity and malnutrition [16,17]. WEPs may contribute a great role in meeting the global attention on addressing malnutrition in all its forms: undernutrition, including micronutrient deficiencies, overweight, and obesity [18]. Indeed, epidemiological and clinical studies advocate the use of plant based diets, including WEPs, as a viable option for the treatment and prevention of overweight and obesity [19].

Typical examples of health promoting components that have been reported to have reduced level in cultivated crops while higher levels in wild counterparts include glucosinolates (GLs) [20,21]. Also, despite their potential health benefits, GLs impart poor palatability to the plants containing them and are thought to be responsible for some nutrient rich wild crops to remain wild. Likewise, certain species in the Capparidaceae/Capparaceae (the caper family) that are adapted to harsh dry climate including *Boscia, Cadaba, Crateva,* and *Maerua*, which form part of a long and deep food tradition in some Sahara regions and which may afford nutritious food, have remained wild as they contain bitter tasting GLs [22]. Such plants may play a vital role in improving food security and promoting health. Plants of the caper family are tropical relatives of the Cruciferae of temperate regions [23], both families being characterized by GLs [24-26]. The caper family is a tropical and subtropical family, which is well represented by woody species in Africa [27] plus high number of wild edible [28] and medicinal [29] species in Ethiopia. The genus *Maerua* comprises about 80 species distributed in the tropical and sub-tropical areas confined to shrubby savanna and semi-desert regions [27], embracing species of trees or shrubs bearing edible, larger fleshy fruits [30]. *Maerua subcordata* (Gilg) DeWolf, a wild food and medicinal plant with a large tuber adapted to low-input agriculture and occurring in the dry parts of East Africa, belongs to the caper family [27]. Therefore, considering the above viewpoints, the present work attempts to provide data on the ethno-medicinal claims of *M. subcordata* by the Kunams of Northern Ethiopia along with relevant literature claim elsewhere supporting its further scientific investigation tailored to reveal its potential utilization as a functional food and/or alternative herbal remedy.

2. Methodology

2.1. Description of study area

Data was collected in Tahitay Adiyabo district, north-west of Tigray, Northern Ethiopia from the Kunama communities who are Nilotic people living in Ethiopia and Eritrea. In Tigray, the

Kunamas form a minor ethnic community who live in the Kafta Humera and Tahitay Adiyabo districts near the border with Eritrea. They are well known for treating human and livestock ailments using herbal medicine and live in remote and isolated areas. In the Tahitay Adiyabo district where data was collected, the Kunamas mainly reside in two sub-districts called Lemlem and Shemblina.

2.2. Ethical statement

The ethical aspects of the study was reviewed and received an expedited ethical approval (ERC 1046/2017) by the Health Research Ethics Review Committee of the College of Health Sciences, Mekelle University.

2.3. Ethnomedicinal data collection and plant authentication

Focus group discussions (FGDs) and oral interviews were used to collect ethno medicinal knowledge and practice by the Kunamas about the study plant locally called '*Ashkulebya*'. The FGDs included mainly traditional healers of both men and women, few local guides and translators that formed a total of twelve to fifteen individuals. To arrange the FGD, ethnic representatives were first approached who described us that the Kunamas in the Tahitay Adiyabo district mainly reside in two sub-districts (Lemlem and Shemblina) and helped us to arrange FGDs in the two sub-districts during the weekend in places where the local people discuss their social issues. Volunteer participants were selected by peer recommendations and oral consent was obtained from each participant before starting the FGD. Interviews were made with selected traditional healers to further enrich the information obtained from the FGD. A plant specimen was collected for authentication from Lemlem sub-district, pressed on a paper and then taken to the National Herbarium at Addis Ababa University, Addis Ababa, Ethiopia where it was authenticated as *Maerua subcordata* (Gilg) De Wolf (Capparidaceae) and a specimen (Voucher number MG001/2007) was deposited.

2.4. Literature review

Data on ethnomedicinal use by the Kunama community was collected referring to its local name, '*Ashkulebya*'. To retrieve further data on this plant, it was authenticated and assigned a scientific name. Then online literature resources accessible *via* the Library of Wageningen University and Research Centre and/or Google databases with the search term "*Maerua subcordata*" were retrieved and systematically reviewed.

3. Results

3.1. Ethnomedicinal use of *M. subcordata* by the Kunamas

M. subcordata produces a large tuber (**Fig 1**) which is well adapted to dry areas. It was also observed in the field that some of its ripe fruits, unless protected by co-grown spiny shrubs such as *Acacia* spp, were found pierced by birds implying that birds may feed on either the fruit mesocarp or seeds of the plant. Informants of the Kunamas stated that *M. subcordata* is among herbal medicines commonly used by the healers. Their traditional practice follow a local custom based disease perception and management. i.e. Members of the community may acquire ethno-medicinal knowledge but only healers are empowered to prescribe remedies because they perceive that herbal remedies are effective when they are collected, prepared and dispensed by the traditional healers; and if done by others, the herbal preparations may not work as remedy or may even be disease aggravating. Variation in traditional knowledge and practice among the healers was also reflected in the two data collection localities as will be described later.

Figure 1. Pictures of *M. subcordata* different parts. Arrows show ripe fruits pierced by birds.

3.1.1. Traditional use of *M. subcordata* by Kunamas at the Lemlem sub-district

The informants described that *M. subcordata* tuber is used for the treatment of malaria. As per their diagnostic specification, malaria symptoms include fever, pain, headache, muscle pain (myalgia), abdominal disturbance (nausea, vomiting, and diarrhea), and abdominal swelling (distended abdomen). Someone with one or more of these symptoms is diagnosed as malaria patient and is given *M. subcordata* tuber or other herbal remedies. They also indicated that use of this plant as a remedy is preferred (i) if the malaria symptoms include muscle pain; and (ii) if there is a fear that other medicinal plants may harm children or pregnant women. In other words, it is the preferred remedy for children and pregnant women. Its preparation and usage as antimalarial remedy is that first, the healer collects the tuberous root, dries and stores it in pottery or wooden containers. Dry season but not rainy season was mentioned as the preferred collection time. When the healer is visited by a patient, an amount (variable and determined by the healer depending on the situation, such as age and gender of patients) of dried root is powdered and mixed with melted clarified butter (traditional ghee) (**Fig 2**). The mixture is then taken orally or applied topically (whole body of patient smeared with the mixture) daily for four days.

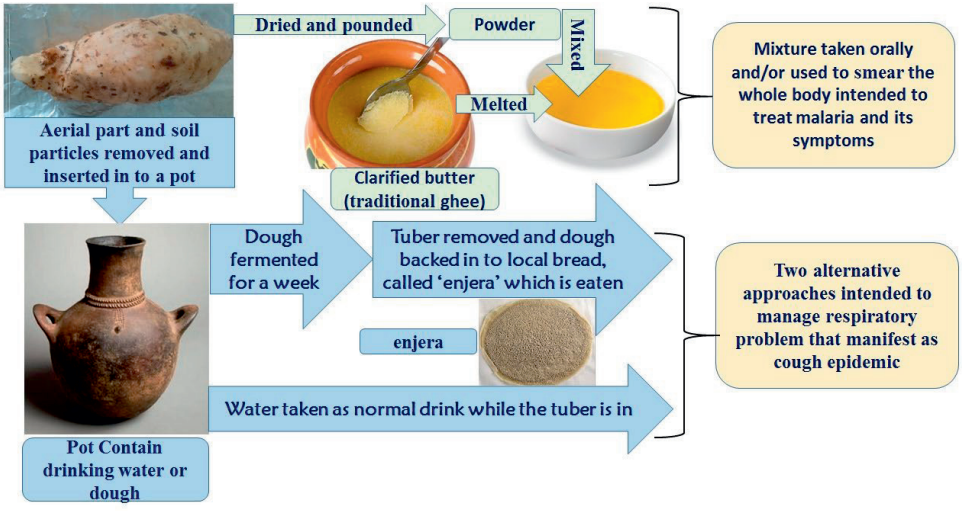

Figure 2. Illustration for mode of ethnomedicinal use of *M. subcordata* tuber by the Kunamas

3.1.2. Traditional use of *M. subcordata* by Kunamas at the Shembilina sub-district

The Kunams at the Shembilina use *M. subcordata* tuber to manage acute respiratory problems that are usually manifested as a seasonal cough epidemic while the leaf is used for wound

healing. As per the informants description, typically in autumn, there is a high possibility of getting illnesses, a cough epidemic being quite common and children being more susceptible. They described this period as characterized by flowering and fruiting of crops and other wild plants as well as several insects hovering over the flowers and wet areas. They perceive that pollen from the flowers or insect-borne rubbles are the cause of cough epidemic, and mainly during such situations, the Kunamas practice various traditional preventive and curative measures including use of herbal medicines, of which one is *M. subcordata.* To manage a cough epidemic with this plant, either of two options of preparation and usage are followed (**Fig 2**). In the first approach, the fresh tuber of the plant is dug out, washed with water to remove soil and then soaked in drinking water in a pot. Every member of the household, with and without cough, drink the water while the tuber is soaked in. This is done for about a week starting from the time of soaking. After a week, if the epidemic continues, the root tuber is replaced by another fresh one. In a second approach, alternatively, the washed tuber is soaked in a dough (usually made from sorghum flour, but flour from other cereals can also be used) for a week. After a week, the tuber is taken out of the dough and the dough baked into a local bread called 'enjera' which is eaten by the patients and all household members. Again this procedure may be repeated for another week, if the epidemic continues. Moreover, the healers and elderly people advise children not to expose themselves to pollen dusts and not to eat unripe or uncooked fruits. For wound healing, dried leaves are thoroughly powdered and a paste is made with water and the paste is smeared on the wound.

3.2. Literature review on *M. subcordata*

In addition to the FGDs and oral interview approaches to collect ethnomedicinal data from the Kunamas, literature was searched on its possible health claims and related ethnobotanical reports elsewhere, which became possible after its authentication. The literature showed that *Maerua subcordata* (Gilg) De Wolf (Syn: *Courbonia subcordata* Gilg, C. *tubulosa* Gilg and Bened) occurs in the dry parts of East Africa, constituting one of 16 *Maerua* species in the flora of Ethiopia and Eritrea [27,31,32]. Its ethnobotanical data were related to three main claims: water purifying agent, food item, and herbal medicine which are detailed in the discussion part. Also, despite scarce phytochemical studies, the quaternary ammonium compounds like stachydrine and 3-hydroxystachydrine were identified in *M. subcordata* [33].

4. Discussion

In the study area, malaria, acute respiratory infections, acute febrile diseases, diarrhoea, helminthiasis, infections of skin and subcutaneous tissue, pneumonia and dyspepsia (impaired digestion) are common illnesses [34]. Among these, *M. subcordata* is used to manage malaria and acute respiratory problems. The Kunamas at the Lemlem sub-district used *M. subcordata* root powder mixed with clarified butter to relief malaria. Sachydrine, identified in the root of this plant by earlier study [33], was shown to exhibit anti-malarial [35] and anti-inflammatory [36-38] activities which may partly support the antimalarial use of the plant. Besides, looking into the detailed practice of the herbal preparation may indicate that active ingredients might also come from the clarified butter that was used to prepare a herbal mixture for antimalarial use. This is because, the clarified butter (traditional ghee) is generally prepared by melting and boiling of butter with different herbs and spices, and then all the ingredients allowed to separate by density. The clarified butter, which may extract some of the constituents of the herbs and spices used to prepare it, is then decanted into another container leaving the curd material in the boiling pan [39]. Therefore, any associated health benefit might come from constituents of *M. subcordata* and/or constituents of the herbs and spices used to prepare the clarified butter. Such preparations may help, at least, to relief symptoms of malaria.

Similarly, the Kunamas at the Shembilina use *M. subcordata* fresh tuber as a component of drinking water or as part of food preparation to manage acute respiratory problems manifested as a cough epidemic. The practice seems to be intended to help the body's defence system and for a dual purpose of prevention and curative because all household members with or without cough take the remedy. Further, they advise children not to expose themselves to pollen dusts and not to eat unripe or uncooked fruits as they associate the cause of the cough with insect-borne infections and/or plant allergens. This claim may make sense since pollen allergens are causes of seasonal cough [40] and the season in which cough epidemics may happen in the study area is a season of flowering and fruiting, which is often accompanied by air breathing loaded with pollen allergens [41,42]. They also mentioned that children are more susceptible indicating the remedy may help to boost the immune system since both potential causes (infection, and plant allergens) of the cough interact with the immune system and children have a relatively immature immune defence [40,43]. Similar use of the root for purposes of improving general body strength and health is reported in Kenya [44] implying possible effect as adaptogen: herbal preparation or natural compound that increases adaptability and survival

of organisms under stress [45]. Considering that glucosinolates are characteristic constituents of many species of the caper family including *Maerua* species [20,24] and that glucosinolates are implicated to promote immune responses [46], which were suggested to be responsible for the adaptogenic effect of certain herbs [47], it may be expected that the herbal preparation may increase the body's ability to resist the damaging effects of stress from infections or plant allergens. Moreover, sachydrine which was detected in this plant part [33], was shown to act as antitussive [48] and hence may have possible contribution to support the claimed effect.

In line with the practice by the Kunamas, the literature data also show a widespread use of the tuber as element of drinking water. Its use to clarify turbid water is well documented, mainly in Ethiopia and Kenya [27,49-53]. In Ethiopia, its use as water purifier is well known over the plain of the Omo river basin and the adjacent areas where the plant is widespread. The whole tuber or slices from it are mixed with turbid water to clarify it. Its water clarifying efficacy was proven by laboratories that revealed its ability to flocculate clay particles of muddy water and its ability to reduce turbidity and microbial load both as primary coagulant and as coagulant aid to alum [49,54]. This practice may imply possible health benefit of the tuber, at least, *via* improving the quality and sanitation of drinking water. Likewise, the literature data show that root and leaf parts of *M. subcordata* are used in ethno-medicine. In Somalia, paste from fresh root is applied on wounds to improve healing and to relief pain; root powder boiled with cow milk and water is taken to treat tonsillitis; water extract of root is used as filtered drops to treat infections of the eye or ear; crushed root mixed with water and honey is taken as abortifacient; and an infusion of the root is used as a laxative [31,32]. In Kenya, root boiled in water is used as anti-helminthic [55] and to treat diabetes, high blood pressure, allergic disorders, microbial infections of blood, to improve appetite, and to induce sleep when taken at a high dose [52]; broth from root is used for purposes of general body strength and health [44]; and leaves are used to treat ophthalmic diseases[56,57] and pneumonia [58]. Also, use of the plant as wild food is reported. In Kenya, Uganda, Sudan, and Ethiopia, fruits are boiled for a meal or part of a meal [59]. In case of Ethiopia, ripe and raw fruits (excluding peel) are used as wild food, both as supplementary and famine food [7]. In Kenya, the plant is source of edible fruits [56]; and root tuber is a famine food which is also chewed to quench thirst in the dry season [44].

So far, very few scientific studies exist that attempt to justify the traditional claims on *M. subcordata*. An aqueous extract of the root was shown to exhibit guinea-pig ileum-contracting effect that was associated to its quaternary ammonium salts and was suggested to support the

traditional use of the root in wound healing [31,33]. Likewise, little work has been done to characterize the nutritional attributes of *M. subcordata*. A report on its root showed high amounts of proteins, polysaccharides (mostly amylopectin), high levels of sodium, potassium and magnesium, but trace levels of iron, manganese, nickel, zinc, and practically no aluminium [60].

Last but not least is safety issue. While considering potential benefits of *M. subcordata*, safety issues should also be taken into account and possible hazard and exposure need be considered to check if the use may present a risk. Uncooked fruits are perceived as toxic and boiling and re-boiling them several times was suggested to render them non-toxic and edible [56]. An additional concern could be that unlike the claim in the study area that *M. subcordata* tuber is relatively safe and hence a preferred remedy for children and pregnant women, reports from Somalia indicate its use as abortifacient [31,32], which if the later claim is true, may point at a possible adverse effect. On the other hand, despite a claim on quaternary ammonium compounds in the root which, if excessively used, may cause a slight intoxication (stomach pain, dizziness, vomiting), these problems were not detected along the area of the Omo river; maybe because the quantities ending up in the water when used to clarify it, were obviously too small to cause negative side-effects [49].

Therefore, the above discussions point out that there seems ethnomedicinal support to suggest further scientific investigations into *M. subcordata* aimed to reveal its potential utilization as a functional food and/or alternative herbal medicine. At the same time, scientific investigations should put equal weight to address safety aspects when considering health benefits of the plant.

5. Conclusion

Existing ethnobotanical and ethnomedicinal data on *M. subcordata* indicate that especially its fruit and roots may have agricultural or medicinal values if developed as functional food or alternative medicines. Therefore, research into the potential nutritional attributes, health benefits as well as possible health risks of the plant could be suggested so as to verify its potential importance as a viable agricultural and/or medicinal resource.

6. Competing interests

The authors declare that they have no competing interests.

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Chapter 3

Effects of *Maerua subcordata* (Gilg) DeWolf on Electrophile-Responsive Element (EpRE)-Mediated Gene Expression *In vitro*

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Abstract

Plant extracts and phytochemicals may prevent chronic diseases *via* activation of adaptive cellular stress response pathways including induction of antioxidant and phase II detoxifying enzymes. The regulatory regions of these inducible genes encode the electrophile-response element (EpRE). This study tested the EpRE induction ability of *Maerua subcordata* (fruit, leaf, root, seed) methanol extracts and selected candidate constituents thereof, identified by liquid chromatography coupled with multistage mass spectroscopy, employing an EpRE luciferase reporter gene assay using hepa-1c1c7 mouse hepatoma cells. A parallel Cytotox CALUX assay using human osteosarcoma U2OS cells was used to monitor any non-specific changes in luciferase activity or cytotoxicity. Results showed that fruit, root, and seed extracts were non-cytotoxic up to a concentration of 30 gram dry weight per litre but the leaf extract exhibited some cytotoxicity and that the leaf (despite some cytotoxicity), fruit, and seed extracts showed strong induction of EpRE mediated gene expression while induction by the root extract was minimal. Selected candidates included glucosinolates, isothiocyanates, and some biogenic amines. Subsequent studies showed that methyl-, ethyl-, isopropyl-, isobutyl- isothiocyanates, and sec-butyl thiocyanate as well as glucobrassicin induced concentration (1-100 µM) dependent EpRE-mediated gene expression while the biogenic amines stachydrine and trigonelline acted as inhibitors of EpRE-mediated gene expression at 100 µM. The identification of glucolepidiin, glucobrassicin, glucocapparin, stachydrine, and trigonelline in all extracts was confirmed using standards and based on multiple reaction monitoring; yet, glucobrassicin level in the root extract was negligible. In conclusion, this study provided a first report on EpRE mediated gene expression effects of *M. subcordata*; and despite detection of different glucosinolates in all extracts, those containing glucobrassicin particularly displayed high EpRE induction. Because EpRE inducers are cytoprotective and potential chemopreventive agents while inhibitors are suggested adjuvants of chemotherapy, results of this study imply that process manipulation of this plant may result in herbal preparations that may be used as chemopreventive agents or adjuvants of chemotherapies.

Key words: Electrophile-responsive element (EpRE), extracts, gene expression, *Maerua subcordata*

1. Introduction

Compelling scientific evidences revealed an inverse relationship between consumption of plant foods rich in some phytochemicals and the risk of several chronic diseases [1-5]. Although the basic protective mechanism has not been clearly explained yet, one suggested mechanism is that phytochemicals activate adaptive cellular stress response pathways. While a multitude of cellular targets are affected by phytochemicals, upregulation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway could be a key factor in the cytoprotective properties of phytochemicals by means of which they promote cellular adaptation [1, 6-9].

Under homeostatic conditions, Nrf2 binds to Kelch-like ECH association protein 1 (Keap1), a cytosolic repressor protein that retains it in the cytoplasm and promotes its proteasomal degradation [8,10]. In the presence of Nrf2 activators (oxidative stress, some chemicals including phytochemicals), the Nrf2 dissociates from Keap1 and translocates in to the nucleus where it assembles to a cis-acting regulatory sequences called antioxidant response element (ARE) or electrophile-response element (EpRE), located in the promoter region of genes encoding various antioxidant and phase 2 detoxifying enzymes and resulting in their expression [8,10]. The main mechanism of Nrf2 activation is Keap1-dependent but Keap1 independent mechanisms such as Nrf2 phosphorylation are also reported [11]. Several reactive cysteine residues in Keap1 function as sensors of cellular redox changes and oxidation/covalent modification of some of these critical cysteine thiols in Keap1 would hamper its role in Nrf2 degradation thereby stabilizing Nrf2 and facilitating its nuclear accumulation [8, 10-12]. It is also worth mentioning that prolonged expression of Nrf2 was shown to protect cancer cells by inducing the metabolism and efflux of chemotherapeutics, leading to both intrinsic and acquired chemoresistance to anti-cancer drugs, an effect that can be regarded as the "dark side" of Nrf2. Thus, Nrf2 inhibitors have been suggested as adjuvant therapies to sensitize cancers with high expression of Nrf2 [13] or as treatment options of chemo- and radio-resistant forms of cancer [14].

In vitro assays are becoming more attractive as screening tools because they are rapid, and they have the potential to reduce the number of animals needed for chemical testing [15,16]. Among cell-based reporter gene assays, luciferase assays are often the method of choice for high-throughput screening for many reasons, most notably the high signal above background inherent to bioluminescence [17,18]. Stably transfected cell lines offer several advantages compared to other *in vitro* systems as they are an excellent aid in defining the mechanisms of action of unknown compounds [15]. *Maerua subcordata* (Gilg) DeWolf (Capparidaceae) is a

wild famine food and medicinal plant mainly grown in the dry parts of East Africa where especially its root tuber and leaf parts have many ethnomedicinal claims including treatment of infectious diseases, malaria, ophthalmic and respiratory problems, allergic disorders, wounds, gastrointestinal disorders, pain, diabetes, and blood pressure. It is also used as a tonic agent, appetizer, as well as to induce sleep (high dose) and abortion [19-25]. Thus, considering the above viewpoints and that various plants are a very rich source of phytochemicals that activate the Nrf2 transcription factor [7,10,26], the aim of the present study was to evaluate, using the EpRE-LUX assay and LC-MS^n metabolic profiling, if *M. subcordata* extracts may activate the Nrf2 pathway and identify possible phytochemicals responsible for the activation.

2. Materials and methods

2.1. Chemicals and reagents

Arecaidine hydrochloride was purchased from Alfa Aesar (Karlsruhe, Germany); N-acetylagmatine from Cayman Chemicals-Europe (Sanbio Uden, the Netherlands); sinigrin potassium salt and glucolepidiin potassium salt were from Extrasynthese (Genay Cedex, France); glucobrassicin potassium salt, stachydrine hydrochloride, and trigonelline hydrochloride were from PhytoLab (Vestenbergsgreuth, Germany); agmatine sulfate, dimethyl sulfoxide (DMSO), glucosinolate hydrolysis products (methyl-, ethyl-, isobutyl-, isopropyl-isothiocyanates, and sec-butylthiocyanate), pipecolic acid, tert-butylhydroquinone (tBHQ), Viscozyme L, and resazurin sodium salt were from Sigma–Aldrich (Schnelldorf, Germany/ Zwijndrecht, The Netherlands). Minimum Essential Medium alpha (α-MEM), Minimum Essential Medium alpha 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12), foetal calf serum (FCS) and Phosphate Buffered Saline (PBS) were from Gibco life technology (Paisley, UK); trypsin, nonessential amino acids (NEAA), and G418 were purchased from Invitrogen Corporation (Breda, The Netherlands).

2.2. Collection, authentication, and processing of plant material

The fruit, leaf, root tuber, and seed parts of *Maerua subcordata* were collected from Shiraro area of Tigray, Northern Ethiopia (**S1 Fig**). This wild plant, sometimes considered as invasive weed, does not raise concerns of endangered or protected species. Collection was made from unreserved and publically open locality where local healers also collect their target medicinal plants and hence no special permission was required to make the collection. The plant was authenticated and a specimen (Voucher number MG001/2007) deposited in the National

Herbarium at Addis Ababa University, Addis Ababa, Ethiopia. The plant parts were sorted and dried in the laboratory of Pharmacognosy, Mekelle University, Mekelle-Ethiopia. The outer thin coating of the underground part (root tuber) was peeled off and the peeled tuber was chopped. The chopped pieces were allowed to dry in an oven at a temperature of 40 ºC for four days. The other parts (fruit, leaf, and seed) were dried in the shade at room temperature and fruits were deseeded (seeds taken out from fruits) after drying. The dried plant materials were packed in plastic bags, and stored at room temperature on shelf until they were transported to Wageningen University, the Netherlands; where they were powdered: each dried plant part was splashed with liquid nitrogen to remove moisture and facilitate powdering, and ground using an analytical miller. Each powdered plant material was mixed well, packed in capped plastic tubes, and stored at -80 ºC until further use.

2.3. Cell lines

Cytotox CALUX and EpRE-LUX cell lines were used in the present study. The cytotox CALUX cells (BioDetections Systems, Amsterdam, The Netherlands) are human osteosarcoma U2OS cells stably transfected with a reporter construct carrying a luciferase reporter gene under transcriptional control of a constitutive promoter. These cells have an invariant luciferase expression and were originally designed to assess cytotoxicity. However, they can also be used to investigate whether stabilisation of the luciferase enzyme is occurring during the exposure to compounds or extracts, a phenomenon that would result in increased luciferase activity without underlying increased expression of the gene. These cells were cultured in DMEM/F12 supplemented with 7.5% (v/v) FCS, and 0.5% (v/v) NEAA [27,28]. EpRE (mGST-Ya)-LUX cells were previously developed based on EpRE sequences from the mouse glutathione-S-transferase Ya (mGST-Ya) gene [27]. These cells are hepa-1c1c7 mouse hepatoma cells stably transfected with a reporter construct carrying a luciferase reporter gene under transcriptional control of an EpRE-enhancer element in conjunction with a minimal promoter and an initiator [29]. The EpRE-LUX cells were cultured in α-MEM medium supplemented with 10% FCS and were maintained at 37 ºC in a humidified atmosphere with 5% CO_2 [27,29]. For both cell lines, 200 µg/ml G418 was added to the culture medium once per week as selection pressure to maintain cells with constructs [27].

2.4. Preparation of extracts from Maerua subcordata

Two types of extracts: non-hydrolysed and enzyme (Viscozyme L) hydrolysed extracts were prepared from powders of *M. subcordata* fruit, leaf, root tuber, and seed samples following the procedure described by Gijsbers *et al*. (2012) [27]. In short, non-hydrolysed extracts were prepared by adding 3.4 ml methanol to 0.6 g powdered plant material followed by 10 min sonication and 15 min centrifugation at 1000 g. Then the supernatant of each sample was filtered using 0.2 µm polytetrafluoroethylene (PTFE)-filters (Whatman, Germany) and freeze-dried after the methanol was evaporated under a stream of nitrogen. Dried extracts were stored at -80ºC until further use. Prior to analysis in the EpRE-LUX assay, the extracts were re-dissolved in DMSO:α-MEM (1:2 v/v). Enzyme hydrolysed extracts were prepared by adding 300 µl sodium acetate (0.1 M, pH 4.8) and 100 µl of Viscozyme L to 0.6 g powdered plant material, followed by 1 hr incubation in a water bath at 37 ºC. Then, samples were put on ice and 3.0 ml of methanol was added to each sample. Subsequently, the samples were sonicated, centrifuged, filtered, dried and stored in the same way as described for the non-hydrolysed extracts. Prior to analysis in the EpRE-LUX assay these extracts were re-dissolved in DMSO:α-MEM (1:4 v/v).

2.5. EpRE-LUX assay

The EpRE-mediated gene expression induction capacity of *M. subcordata* methanol extracts, selected individual compounds, tBHQ as positive control, and DMSO as solvent control was evaluated by measuring luciferase activity in EpRE-LUX reporter cells. The assays were performed essentially as described by Gijsbers *et al*. (2012) [27]. Briefly, EpRE-LUX cells were seeded in the 60 inner wells of a white 96-well view plate at a density of 20,000 cells per well in 100 µl culture medium. 200 µl PBS was added to the outer 36 wells to maintain physical homogeneity throughout the plate. The seeded cells were incubated for 24 hr to allow them attach and form a confluent monolayer. Then, the culture medium was carefully removed and, instantly, the cells were exposed to 200 µl α-MEM (without FCS and antibiotics) containing test samples for another 24 hr. The extracts were dissolved in DMSO:α-MEM (1:2 v/v) while tBHQ and the other individual compounds were dissolved in DMSO. The final concentration of DMSO in the exposure medium was 0.5% for studies with individual compounds whereas for studies with extracts, up to 1.5% was used after it was checked that 1.5% DMSO had no effect on cell viability compared to 0.5%. On each plate, 10 µM tBHQ was included as a positive control. After 24 hr exposure, medium was removed, cells were washed with ½ PBS

(PBS half diluted with Nano pure water), and exposed to 30 µl low salt lysing buffer (1.2114 g Tris, 0.084 g dithiothreitol, 0.7287 g 1,2-cyclohexylenedinitrilotetraacetic acid; in a litre of Nano pure water, pH 7.8). Then plates were covered by aluminium foil, placed on ice for 15 minutes and frozen overnight at -80 ^{o}C. Then the plates were thawed and luciferase activity per well in the lysate was measured in relative light units (RLU) using a luminometer (GloMax-Multi Detection System–Promega) and a flash mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4.7H_2O$, 0.1 mM EDTA, 2.0 mM dithiothreitol, 470 µM luciferine, 5.0 mM ATP; in a litre of Nano pure water, pH 7.8). Results were expressed as fold induction compared to the solvent control. Extracts or compounds giving twofold or greater luciferase activity at a concentration that could be tested without cytotoxicity were considered able to induce EpRE-mediated luciferase expression.

2.6. Cytotox CALUX assay

To each EpRE-LUX assay, a parallel test was done using the U2OS cytotox CALUX cells. These cells were seeded in the 60 inner wells of a white 96-well view plate at a density of 10,000 cells per well in 100 µl assay medium: DMEM/F12 supplemented with 7.5% (v/v) FCS, and 0.5% (v/v) NEAA. The outer wells were filled with 200 µl PBS. The next day, 100 µl medium containing the test samples was added to each well resulting in 200 µl assay medium per well. After 24 hr of exposure, luciferase activity was measured in the same way as in the EpRE-LUX assay described above.

2.7. Alamar blue (resazurin) assay

Cytotox CALUX and EpRE-LUX cells were cultured in 96 well plates in their appropriate medium, described above, for 24 hrs and then the cells were exposed to test samples for another 24 hrs. Then alamar blue (resazurin) reagent solution (10% w/v in PBS) was added directly to the cells (10% v/v i.e. 20 µl reagent to 200 µl cells in medium). Following reagent addition, plates were covered by aluminium foil and incubated for 1 hrs after which fluorescence was measured (λex 580 nm/λem 610 nm) using a plate reader (Molecular Devices, Spectra Max M2) equipped with Softmax Pro software.

2.8. LC−MS^n based metabolic profiling of *M. subcordata*

Liquid chromatography (LC) coupled with multistage mass spectroscopy (MS^n) was applied to identify metabolites in methanol extracts from fruit, leaf, root, and seed of *M. subcordata*. For initial qualitative analysis, extracts were prepared by adding 400 µl of methanol (with 1% v/v

formic acid) to 15 mg powder of each plant material. The mixture was vortexed, sonicated for 15 minutes, and centrifuged at 1000 g for 10 minutes. The supernatant was filtered using 0.2 µm polytetrafluoroethylene (PTFE)-filter (Whatman, Germany) and stored at -80 ^{o}C until used. The extracts were subjected to LC-MS^{n} analysis based on coupling of ion-trap MS with Orbitrap Fourier transform MS. To support structural characterization of the many metabolites present in such complex samples, Ridder *et al.*, (2012) [30] offered a novel method (http://www.emetabolomics.org/magma) to automatically process and annotate LC−MS^{n} data sets on the basis of candidate molecules from chemical databases. In 'Ms Annotation based on in silico Generated Metabolites' (MAGMa), which is an online application software, uploaded spectral data are automatically annotated with hierarchical trees of in silico generated substructures of candidate molecules, retrieved from PubChem and from a subset of PubChem compounds present in Kegg. Alternative structures of candidates are ranked on the basis of calculated matching score and displayed on the user interface [30,31]. The most likely candidate structures that could be possible constituents of the extracts were manually selected from the MAGMa user interface. The identity of selected candidates was further confirmed and quantified by LC-MS/MS based on multiple reaction monitoring (MRM) and calibration curves from standards. The identity of some candidates was further confirmed and quantified by standard calibration curves as described below.

2.9. Confirmation of selected candidates by LC-MS/MS

This later analysis targeted mainly glucosinolates and extraction was done using a modified ISO9167-1 method as described by Ishida *et al*. (2011) [32] and Doheny-Adams *et al*. (2017) [33]. Briefly, into each test tube, 5 ml of 80% v/v methanol : water (buffered with 1% v/v formic acid) at room temperature (≈20 ºC) was added to 100 mg (dry weight) of powdered sample. The mixture was then vortexed and left for 30 min at room temperature. Then, it was ultra sonicated for 15 minutes and centrifuged at 1000g for 10 minutes. Supernatant was filtered (0.2 µm PTFE-filter) and stored at -80 ^{o}C until used. LC-MS operating conditions were optimized using standard compounds and the extracts and the different concentrations of standard solutions for calibration curves were analysed under identical conditions. The MS analysis was performed in Multiple Reaction Monitoring (MRM) mode on LCMS-8040 (Shimadzu, Japan) triple quadrupole with Electrospray Ionization source operated in negative ion mode for glucosinolate analysis and positive ion mode for analysis of biogenic amines. The compounds of interest in the extracts were identified by their retention time and LCMS-MRM

characteristics and quantified based on equations from calibration curves. Ares *et al* (2016) [34] used sinigrin as an external standard to quantify glucosinolates from broccoli where individual standards were not available. Likewise, sinigrin was used as external standard to quantify glucocapparin in *M. subcordata* extracts since a glucocapparin standard was not available.

2.10. Data analysis

For each experiment, at least three independent repetitions were performed. Graphs generated from an average of repetitions are presented. Data were analysed using Microsoft Excel 2010/2016, expressed as fold induction over the solvent control, and presented as mean values ± standard error of the mean (SEM). Each data point was measured, at least, in triplicate. Statistical significance was assessed using SPSS 22, applying paired samples statistics t-tests and a significance cut-off value of $p \leq 0.05$.

3. Results

3.1. Effect of *M. subcordata* methanol extracts on cell viability

The resazurin assay results (**fig 1**) show that the screened extracts up to a concentration of 30 gDW/L (gram dry weight per litre) cause no major effect on cell viability of both cell lines, except for the non-hydrolysed leaf extract that exhibited some cytotoxicity (68% and 82% remaining resazurin metabolic reduction activity for EpRE and U2OS cells, respectively). Also, testing with varying concentrations showed reproducible induction of EpRE mediated gene expression by the extracts (**fig 2**) and the highest non-toxic concentration (30 gDW/L) of all extracts, except the leaf extract, was used as a screening concentration for all extracts so as to make comparison among them.

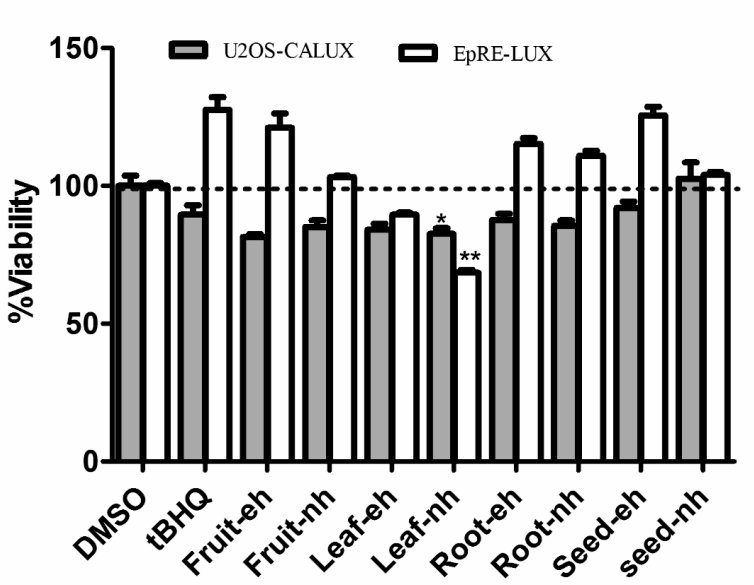


Fig 1. Resazurin assay results showing effects of enzyme hydrolysed (eh) and non-hydrolysed (nh) methanol extracts of different parts of *M. subcordata* at 30 gDW/L and tBHQ (10 μM) on cell viability. Data are presented as mean ± SEM of six replicates. Viability of cells exposed to the solvent control (1% DMSO) was set at 100% and asterisk indicate a significant difference from the solvent control: *$p < 0.01$; **$p < 0.0001$.

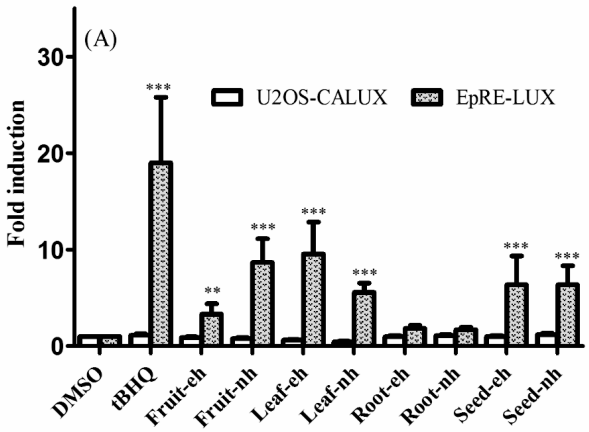
3.2.Induction of EpRE mediated luciferase expression by *M. subcordata* methanol extracts

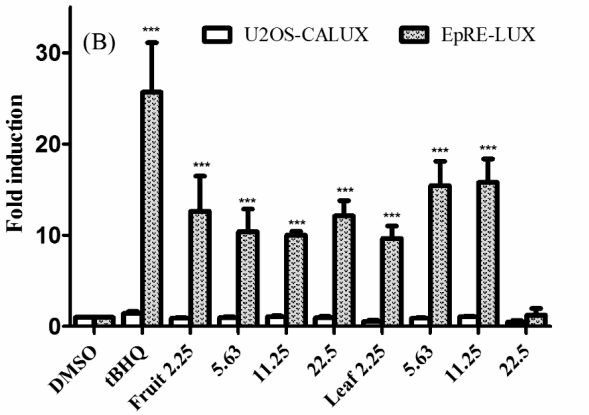
The EpRE assay results show that both enzyme hydrolysed (eh) and non-hydrolysed (nh) methanol extracts from the fruit, leaf, and seed materials as well as tBHQ (positive control) exhibited a high increase in luciferase activity, whereas the induction by the root extracts was minimal as compared to the solvent control (DMSO) (**fig 2A**). Unlike to what was expected, enzyme hydrolysis did not increase luciferase activity although enzyme hydrolysis seems to decreased the cytotoxicity of the leaf extract as the nh leaf extract was more cytotoxic than the eh leaf extract. Further assays with different concentrations of the non-hydrolysed extracts reproduced EpRE induction by the fruit, leaf, and seed extracts while the root extract showed some induction at lower concentrations that declined with increasing concentrations (**fig 2B&C**). These second round assays, which were performed along with candidate compounds, revealed that unlike the candidates that showed concentration dependent increase in luciferase

activity (**fig 3**), the extracts failed to maintain concentration dependent increase in luciferase activity. Moreover, although the maximum concentration for the fruit and leaf extracts was lowered to minimize cytotoxicity, the fruit extract was still non-cytotoxic but the leaf extract was even more cytotoxic at 22.5 gDW/L than previous assays done at 30 gDW/L implying high variability of the leaf extract both in cytotoxicity and induction of EpRE-mediated gene expression. Based on these observations the present report focused on the intrinsic properties of the extracts that the fruit, leaf, and seed exhibited reproducible high EpRE induction while induction by the root was minimal.

To monitor non-specific interference of luciferase by the extracts, a parallel test was done using the U2OS-CALUX assay. In line with minor effects on cell viability in the resazurin assay for the leaf extracts, the U2OS assay also shows that the leaf extracts exhibited some cytotoxicity (remaining luciferase activity of 62% and 44% compared to the solvent control for enzyme hydrolysed and non-hydrolysed, respectively) while the fruit, root, and seed extracts showed similar activity as the solvent control indicating the absence of cytotoxicity again in line with the resazurin assay. Since luciferase activity at non-cytotoxic concentrations in the U2OS-CALUX assays were similar as in the solvent controls, it was concluded that the extracts did not interfere with luciferase itself.

For fruit extracts, the non-hydrolysed extract showed greater EpRE induction than the enzyme hydrolysed while both type of seed extracts showed comparable induction. For the leaf, the enzyme-hydrolysed extract showed greater EpRE induction than the non-hydrolysed which could be due to a lower number of viable cells (44%) in the assay with the relatively higher cytotoxic non-hydrolysed extract. Although the leaf extracts were tested at levels that may induce some cytotoxicity, using the same concentration of 30 gDW/L allowed better comparison to the extracts from other plant parts to characterize their intrinsic properties towards EpRE-mediated gene expression.


(A)
U2OS-CALUX
EpRE-LUX
Fold induction
30
20
10
0
***
**
DMSO
tBHQ
Fruit-eh
Fruit-nh
Leaf-eh
Leaf-nh
Root-eh
Root-nh
Seed-eh
Seed-nh


(B)
U2OS-CALUX
EpRE-LUX
Fold induction
30
20
10
0
***
DMSO
tBHQ
Fruit 2.25
5.63
11.25
22.5
Leaf 2.25
5.63
11.25
22.5

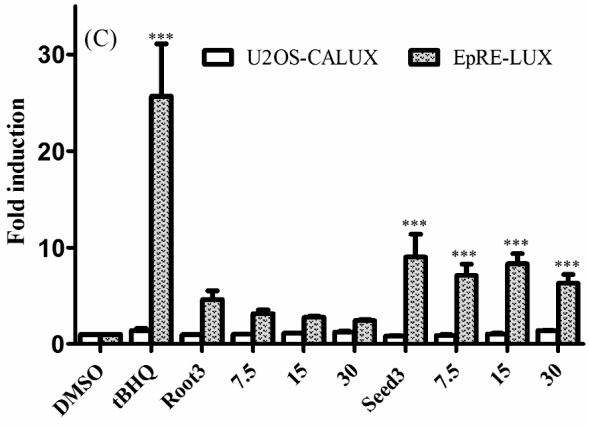
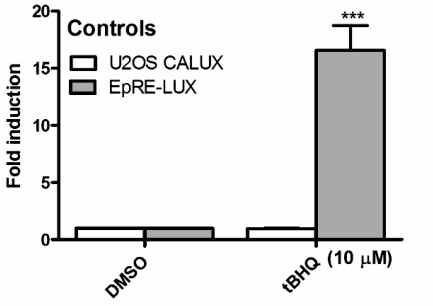
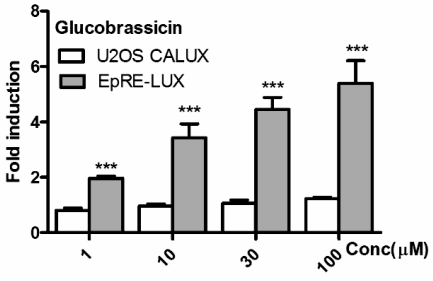

(C)
U2OS-CALUX
EpRE-LUX
Fold induction
30
20
10
0
***
DMSO
tBHQ
Root3
7.5
15
30
Seed3
7.5
15
30

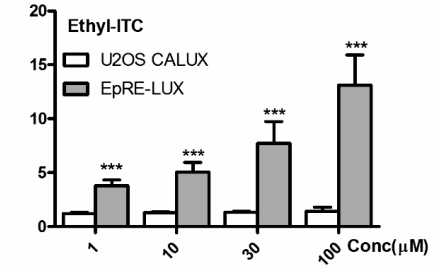
Fig 2. Luciferase activity in U2OS-CALUX and EpRE-LUX cells after 24 hr exposure to *M. subcordata* methanol extracts. (A) Enzyme hydrolysed (eh) and non-hydrolysed (nh) extracts at 30 gDW/L, different concentrations of (B) fruit and leaf nh extracts, and (C) root and seed nh extracts. Luciferase activity is expressed as fold induction compared to solvent control. tBHQ (10 μM) was used as the positive control. Data are presented as mean ± SEM of three independent experiments, each done in six replicates. Asterisks indicate a significant difference from the solvent control: $^{**}p < 0.01$; $^{***}p < 0.0001$.

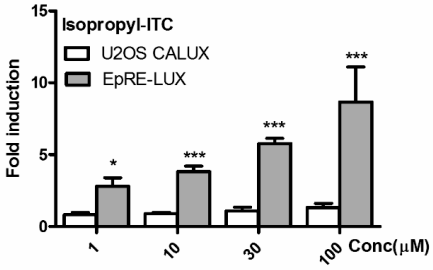
3.3. Induction of EpRE mediated luciferase expression by candidate compounds of *M. subcordata*

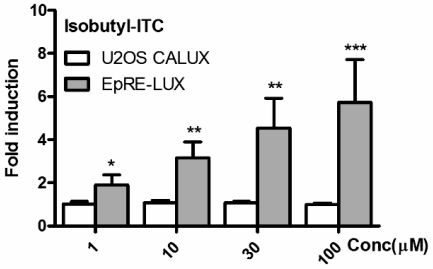
To define the main phytochemicals responsible for the observed induction of EpRE mediated gene expression, LC-MS^n metabolic profiling was done on the methanol extracts. As enzyme hydrolysis did not influenced EpRE induction by the extracts to a large extent, only the non-hydrolysed samples were analysed. The LC-MS^n data resulted in a tentative identification of various constituents including glucosinolates (**S2 Fig)** and some biogenic amines (**S3 Fig)** to which focus was given as possible EpRE inducers. The glucosinolates were detected in negative ion mode while most biogenic amines in positive ion mode. Except for glucobrassicin, the respective isothiocyantes of the detected glucosinolates were screened for their EpRE mediated gene expression induction capacity because of their commercial availability. Thus, glucobrassicin, methyl-, ethyl-, isopropyl-, isobutyl- isothiocyanates, and sec-butyl thiocyanate which are hydrolysis products of glucocapparin, glucolepidiin, glucoputranjivin, isobutyl glucosinolate, and glucocochlearin respectively as well as some biogenic amines were tested in different concentrations (1 - 100 μM) for their EpRE mediated gene expression induction capacity. The results (**fig 3)** showed that glucobrassicin, the isothiocyanates and *sec*-butyl isocyanate exhibited a concentration-dependent increase in luciferase activity compared to the solvent control. Conversely, the biogenic amines showed either no activity or a slight inhibitory effect on luciferase activity. The biogenic amines agmatine sulfate, N-acetylagmatine**,** anthranilic acid, pipecolic acid, and arecaidine hydrochloride were inactive up to a concentration of 100 μM while stachydrine and trigonelline showed inhibition (**fig 4**) of EpRE mediated gene expression at the highest concentration tested (100 μM). None of these chemicals affected luciferase signal in the U2OS-CALUX assay up to the highest concentrations tested, indicating that they do not interfere with luciferase itself (**figs 3 and 4**).

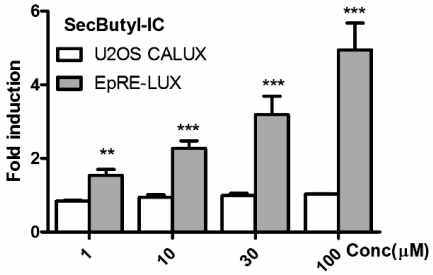

Controls
U2OS CALUX
EpRE-LUX
Fold induction
DMSO
tBHQ (10 μM)


Glucobrassicin
U2OS CALUX
EpRE-LUX
Fold induction
Conc(μM)


Ethyl-ITC
U2OS CALUX
EpRE-LUX
Conc(μM)


Isopropyl-ITC
U2OS CALUX
EpRE-LUX
Fold induction
Conc(μM)


Isobutyl-ITC
U2OS CALUX
EpRE-LUX
Fold induction
Conc(μM)


SecButyl-IC
U2OS CALUX
EpRE-LUX
Fold induction
Conc(μM)

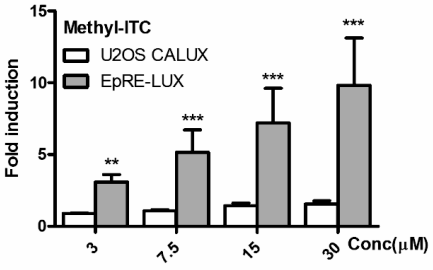

Methyl-ITC
U2OS CALUX
EpRE-LUX
Fold induction
Conc(μM)

Fig 3. Induction of luciferase activity in U2OS and EpRE cell lines after 24 hr exposure to glucobrassicin, ethyl-, isopropyl-, isobutyl-, and methyl-isothiocyanates (ITCs), and sec-butyl isocyanate (IC). Luciferase activity is expressed as fold induction compared to the solvent control and data are presented as mean ± SEM of four independent experiments, each performed in triplicate. Asterisks show a significant difference from the solvent control: *p < 0.05; **p < 0.01; ***p < 0.0001.

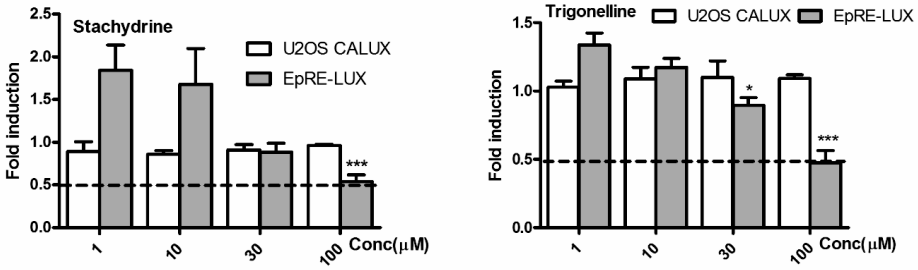


Fig 4. Luciferase activity induction in U2OS-CALUX and EpRE-LUX cell lines after 24 hr exposure to stachydrine and trigonelline. Luciferase activity is expressed as fold induction compared to solvent control. Data are presented as mean ± SEM of four independent experiments, each performed in triplicate. Asterisks show a significant difference from the solvent control: *p < 0.05; ***p < 0.0001.

Further definite identification and quantification (**Table 1**) was made for glucolepidiin, glucobrassicin, stachydrine, and trigonelline by LC-MS/MS-MRM using standard calibration curves based on commercially available reference compounds. Moreover, glucocapparin was quantified using sinigrin as external standard[34] since commercial reference glucocapparin was not available.

One can speculate from **Table 1** that the glucosinolates, mainly glucocapparin may generate concentrations of matching ITCs that could explain a large part of the EpRE induction by all extracts including the root extract, if most would be hydrolysed to these ITCs. However, the root extract did not induce EpRE mediated gene expression.

Table 1. Some glucosinolates and biogenic amines from *M. subcordata* extracts identified and quantified by LC-MS/MS MRM and standard calibration curves. (a) Amount described in microgram per gram dry weight (µg/gDW) and (b) extrapolated concentration (µM) in 96 well plate as calculated from a concentration of 30 gDW/L as applied in the *in vitro* studies with plant extracts. Rt, retention time.

Samples	**Compounds**									
	Glucocapparin		Glucolepidiin		Glucobrassicin		Stachydrine		Trigonelline	
Rt (min)	1.00		1.50		6.95		1.10		1.10	
	a	b	a	b	a	b	a	b	a	b
Fruit	154.69	13.92	1.44	0.12	2.07	0.138	2602.36	545.25	7.59	1.66
Leaf	539.61	48.56	8.86	0.76	28.72	1.921	3290.43	689.42	7.44	1.63
Root	390.60	35.15	13.05	1.13	0.06	0.004	1507.58	315.87	5.41	1.18
Seed	432.80	38.95	3.79	0.33	17.48	1.169	2710.23	567.85	6.53	1.43

Another speculative view could be a possible antagonism by stachydrine as the extrapolated concentration of which is above 100 µM in all extracts. Yet, its level in the root is lower than in the other extracts. Therefore, despite the identification of components in the extracts at seemingly substantial levels that can influence EpRE mediated gene expression, it appears to be difficult to indicate the specific phytochemicals responsible for the EpRE induction by the extracts based on the current data. Further consideration of the complex interactions among multiple components in the extracts, the unpredictable kinetics of glucosinolate hydrolysis during the *in vitro* EpRE induction studies, and the contribution, on the overall effect, of other chemicals in the extracts that were not included in the chemical identification of the present study may be required. In this regard, an activity-directed analysis with fractions and sub-fractions could be of help to identify the phytochemicals with the largest effects. Interestingly, the fruit, leaf, and seed extracts that showed apparently high levels of glucobrassicin exhibited significant induction of EpRE mediated gene expression while the root extract which showed negligible glucobrassicin level, showed insignificant induction. This may indicate that glucobrassicin and possibly other related glucosinolates could be largely responsible for the induction of EpRE mediated gene expression exhibited by *M. subcordata* extracts.

4. Discussion

The present study evaluated the EpRE mediated gene expression induction potential of *M. subcordata* methanol extracts and selected candidate compounds thereof, using an *in vitro* luciferase reporter gene assay. It was shown that the fruit, leaf, and seed extracts revealed strong induction of luciferase activity at 30 gDW/L while the induction by the root extract was less than twofold. To ensure that the induced increase of luciferase activity is not due to luciferase stabilization caused by non-specific or off-target interactions [17,18,35], a cytotox CALUX assay based screening was done parallel to each test with the EpRE-LUX assay [27,28]. Results of this counter screening showed luciferase activity of more or less similar to that of the solvent control for the fruit, root, and seed extracts at 30 gDW/L suggesting the absence of non-specific interference with the luciferase reporter protein. Significantly lower luciferase activity was shown by the leaf extract, which was in line with the reduction of cell viability as measured by the resazurin assay. The remaining cells exposed to the leaf extract at 30 gDW/L still showed a strong induction of luciferase activity in the EpRE assay. Enzyme hydrolysis with Viscozyme L was shown to improve both extraction and biological activity of phenolic compounds [27,36]. Expecting that *M. subcordata* may contain such constituents, enzyme hydrolysed (eh) extracts were prepared along with non-hydrolysed (nh) extracts. However, eh extracts did not show higher EpRE induction compared to nh extracts implying that the components responsible for EpRE induction by the extracts were not substrates of Viscozyme L. Indeed, results from the phytochemical analysis of the extracts revealed the presence of glucosinolates, known EpRE inducers, which are not substrates of Viscozyme L. Yet, enzyme hydrolysis seems to reduce cytotoxicity of the leaf extract (remaining viable cells luciferase activity of 62% and 44% for eh and nh, respectively) and hence a greater induction by the eh extract than the nh extract.

Liquid chromatography coupled with multistage accurate mass spectrometry($LC-MS^n$) can generate comprehensive spectral information of metabolites in crude extracts which contain complex multiple components. In the present study, structural characterization of metabolites in *M. subcordata* methanol extracts was done by $LC-MS^n$–MAGMa structural annotation as described by Ridder *et al.*, [30,31] that resulted in the tentative identification of many compounds including glucosinolates (GLs) and biogenic amines. Moreover, supporting literature data show that alkyl and indolyl GLs are constituents of many species of the Capparidaceae family including *Maerua* species [37]. There is no previous study reporting GLs in *M. subcordata*, but glucobrassicin, glucocapparin, and glucocleomin were reported in other

Maerua species [38-40]. However, glucocleomin was not detected in any of the analysed extracts in the present study. Mainly, glucocapparin is widely distributed throughout the Capparidaceae [38,41] usually as the most abundant compound [40,42-45]. Likewise, glucocapparin was detected in the present study in all extracts of *M. subcordata* in apparently high amounts (**Table 1**) indicating that it also constitutes a major GL in *M. subcordata*.

Because GLs and their hydrolysis products are known inducers of Nrf2, they have been given focus as likely components responsible for the luciferase activity induction by *M. subcordata* extracts. Plants producing GLs possess a β-thioglucosidase, called myrosinase. GLs are stored in vacuoles of plant cells while myrosinases occur compartmentalized in separate but adjacent myrosin cells. Upon plant tissue damage, GLs get into contact with myrosinase at the damage site and hydrolysis is catalysed resulting in various products including isothiocyanates (ITCs), thiocyanates, and nitriles depending on reaction conditions such as pH and temperature. Hydrolysis at neutral conditions or pH 5-7 typically results in the formation of ITCs which are responsible for the biological activities of GLs [46-48]. GLs and their hydrolysis products, mostly ITCs, have long been known for their allelopathic, bacteriocidal, fungicidal, and nematocidal properties while recently, they attracted intense research interest because of their cancer chemoprotective attributes presenting a promising group of natural anti-infective and anti-cancer agents [37, 46, 48-50]. The interest on these unique phytochemicals with versatile biological properties have been more intensified after the discovery that the ITC, sulforaphane, potently induces mammalian cytoprotective proteins through the Keap1–Nrf2 pathway. Nowadays, it seems established that ITCs are well known to target Keap1 for activating Nrf2 pathway resulting in induction of gene expression of antioxidant and Phase II detoxifying enzymes [46,51-54] by means of which they provide numerous health benefits including chemoprevention [55], neuroprotection [56], alleviation of obesity and insulin resistance [57]. Therefore, the GLs and their hydrolysis products that also demonstrated strong concentration-dependent luciferase activity in the present study, might be responsible for the observed induction of EpRE mediated gene expression by *M. subcordata* methanol extracts and may contribute, at least partly, to justify the various traditional medicinal claims on this plant.

Stachydrine and trigonelline were among compounds identified in *M. subcordata*, of which stachydrine was most abundant (**table 1**). The identification of stachydrine in *M. subcordata* supports an earlier study which reported tetramethylammonium, prolinebetaine ethyl ester, stachydrine, 3-hydroxystachydrine and/or 4-hydroxystachydrine (betonicine), 3-hydroxy-1,1-

dimethylpyrrolidinium, and glycine betaine in *M. subcordata* (*Syn: Courbonia subcordata*) [58]. Also, it was shown that stachydrine and 3-hydroxystachydrine along with GLs characterize the family Capparidaceae [58-60]. Trigonelline is a known inhibitor of Nrf2 mediated gene expression [61,62] and results (**fig.4**) of the present study support these reports. Stachydrine also showed a similar trend and hence both compounds were regarded as inhibitors of EpRE mediated gene expression, although at relatively high concentrations (100 μM). This implies the co-existence of constituents in *M. subcordata* methanol extracts with potential competing effects on EpRE induction. i.e. GLs and their hydrolysis products were shown to be inducers while stachydrine and trigonelline, acted as inhibitors.

In line with previous studies that GLs and ITCs are potent activators of Nrf2 [63], EpRE induction by GLs and ITCs in the present study was, at least, ten times more potent than the inhibition by biogenic amines which may imply that in the presence of combined potent inducers and weak inhibitors, the effect of potent inducers may predominate. This could be one possible reason for the fruit, leaf, and seed extracts of *M. subcordata* which showed a higher net EpRE induction despite the presence of substantial amount of less potent inhibitors such as stachydrine. In addition, since aromatic ITCs are generally less volatile than aliphatic ITCs [64] relatively stable induction may be expected from the former. Thus, induction of EpRE mediated gene expression was evident by the fruit, leaf and seed extracts containing apparently substantial amounts of glucobrassicin (aromatic glucosinolate) despite the presence of different aliphatic glucosinolates in all extracts including root extract which showed some induction that tend to decline with increasing concentration. The unpredictable kinetics of glucosinolates hydrolysis and variation in volatility of ITCs may also contribute to the fluctuation of EpRE induction with increasing concentration of extracts (**fig 2B&C**). Yet, the multicomponent and complex nature of the extracts is a great challenge to make sufficient justification because the complexity of plant extracts may be very problematic in maintaining assay integrity. Cell based assays with plant extracts often fail to fulfil assay requirements like high reproducibility, accuracy, and robustness as plant extracts may contain interfering compounds that co-exist with bioactive compounds [65]. Such possible interference was highly reflected by the leaf extract which showed high variability in cytotoxicity and induction of EpRE-mediated gene expression even at the same concentration assayed in different times. Despite these challenges the present study revealed the intrinsic potential of *M. subcordata* extracts to exert potential beneficial effects *via* induction of EpRE-mediated gene expression.

To sum up, the present study demonstrated that *M. subcordata* contains bioactive metabolites typically glucosinolates and biogenic amines which are considered important nutraceutical agents and hence the plant may be a good sources of a noble food product. The glucosinolartes are known inducers of antioxidant and detoxifying enzymes while some of the biogenic amines demonstrated inhibitory effect which may imply that process manipulation of the plant is required to get enriched inducer or inhibitor product. Under assay conditions of possible additive and/or antagonistic effects and complex kinetic interactions among multicomponent extracts, the fruit, leaf, and seed extracts revealed a net strong induction of EpRE mediated gene expression while the root extract showed a net absence or weak induction.

5. Conclusion

This study is the first to demonstrate the EpRE mediated gene expression induction capacity of *M. subcordata*. Especially, the fruit, leaf, and seed extracts exhibited substantial induction while the induction by the root extract was less than twofold at 30 gDW/L. LC-MS^n metabolic profiling of the plant revealed the presence of glucosinolates, which are known inducers, and some biogenic amines such as stachydrine and trigonelline that demonstrated as inhibitors. EpRE inducers are cytoprotective and potential chemopreventive agents while inhibitors are suggested adjuvants of chemotherapy implying that processing manipulation of this plant may result in herbal preparations or functional food products that may be used as chemopreventive agents or adjuvants of chemotherapies.

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Appendix: Supporting Information

Supporting materials can be found at the end of this chapter

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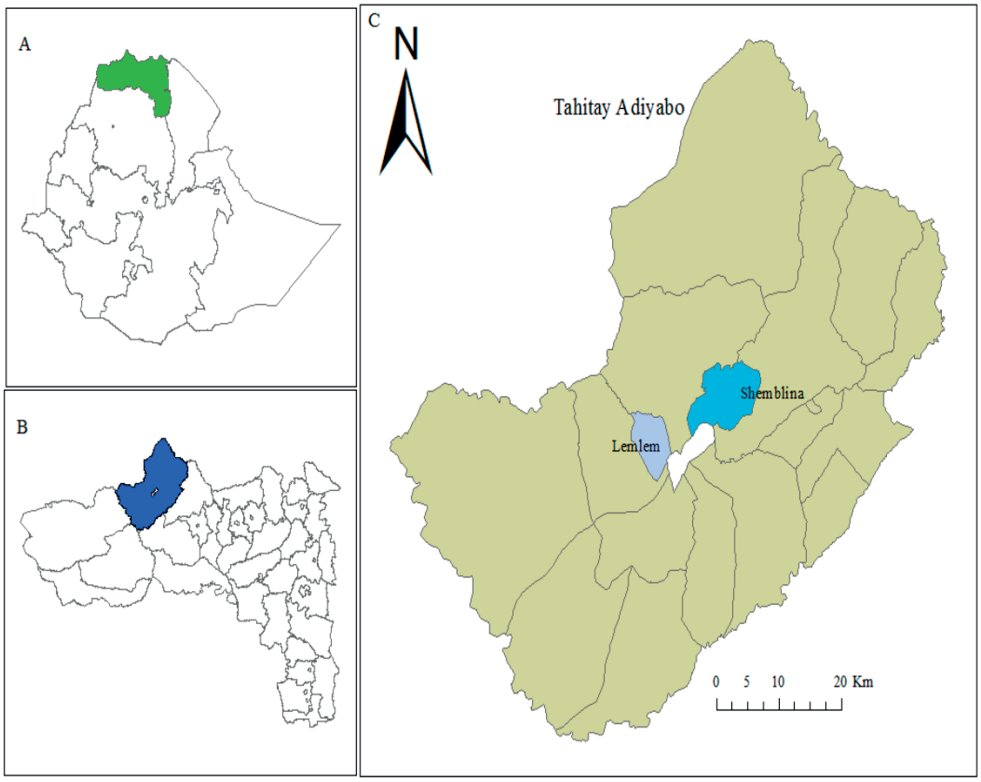
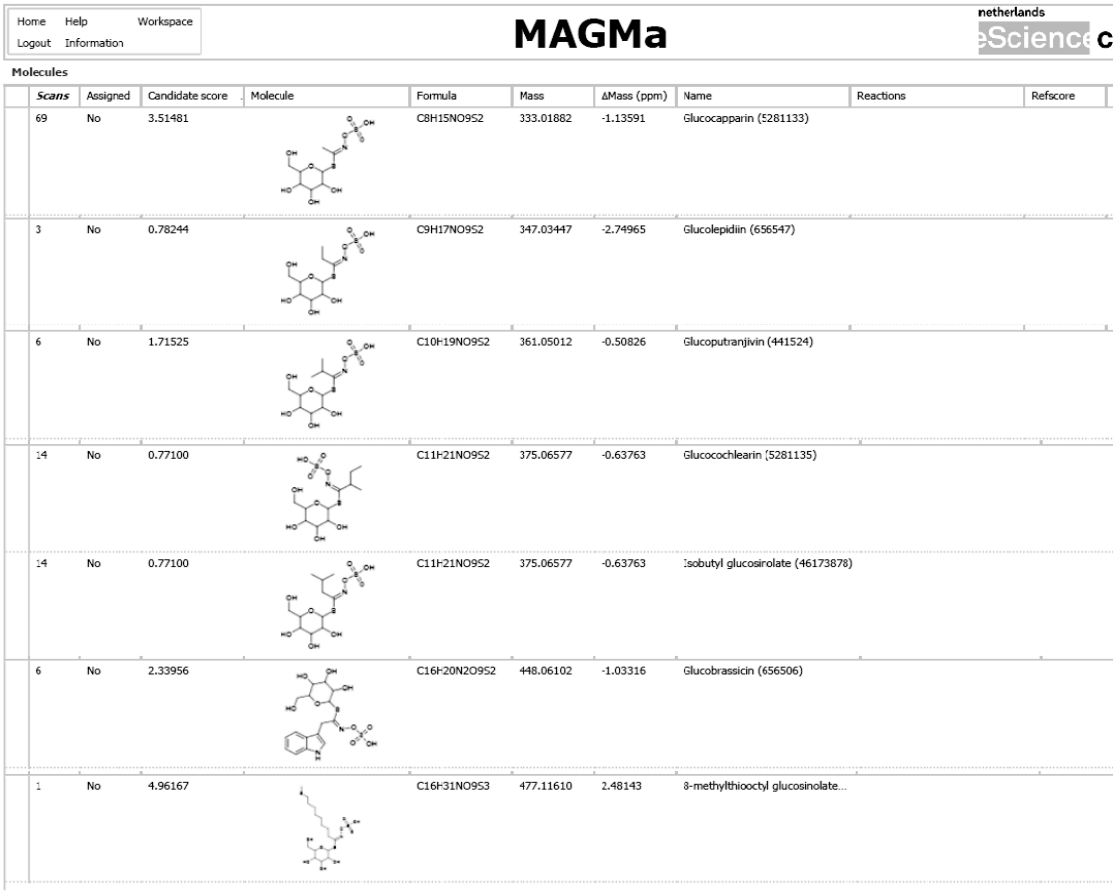
Appendix: Supporting Information

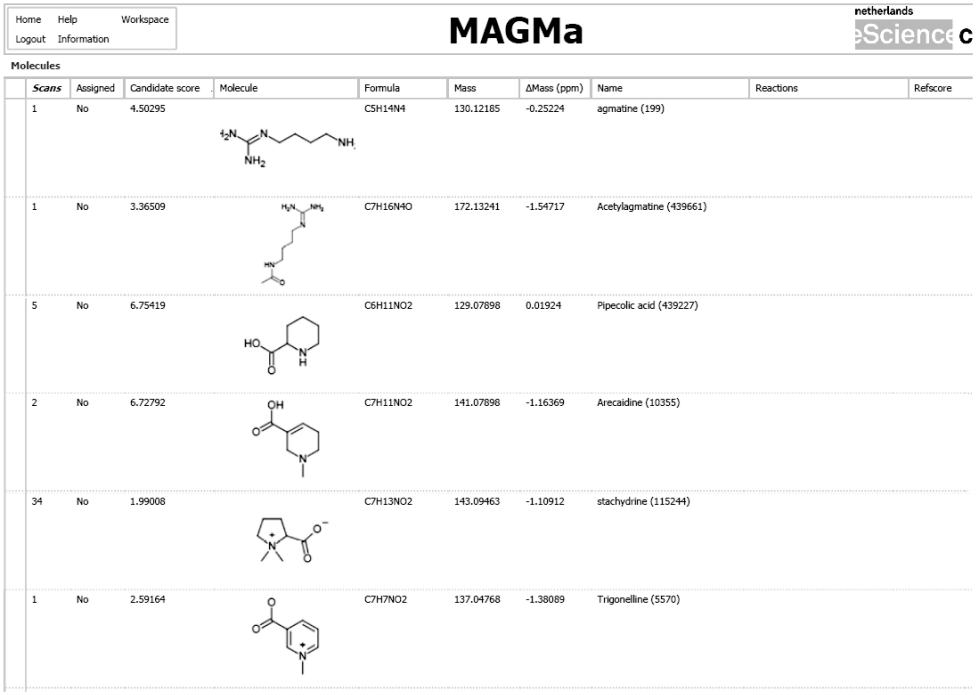
S1 Fig. Map of the plant collection area. (A) location of Tigray region (shaded green), in Ethiopia (B) location of the district (shaded blue) in Tigray, and (C) the localities of plant collection.



S2 Fig. Glucosinlates tentatively identified in *M. subcordata* methanol extracts as taken from the display on MAGMa interface.



S3 Fig. Some biogenic amines tentatively identified in *M. subcordata* methanol extracts as taken from the display on MAGMa interface.



Chapter 4

Induction of Peroxisome Proliferator Activated Receptor γ (PPARγ) Mediated Gene Expression and Inhibition of Induced Nitric Oxide Production by *Maerua subcordata* (Gilg) DeWolf

Mebrahtom Gebrelibanos Hiben, Laura de Haan, Bert Spenkelink, Sebastiaan Wesseling, Jacques Vervoort, Ivonne M.C.M. Rietjens

Submitted for publication

Abstract

The disease preventive and therapeutic potential of botanicals is linked to their multicomponent phytochemicals that often exert pleiotropic effects *via* targeting multiple molecular signaling pathways. The peroxisome proliferator-activated receptors (PPARs) are transcription factors that control metabolic homeostasis and inflammation. The nuclear factor kappaB (NF-κB) is a master regulator of inflammatory genes like inducible nitric-oxide synthase that result in nitric oxide (NO) overproduction. The present study tested the induction of PPARγ mediated gene expression in U2OS-PPARγ cells employing luciferase reporter gene assay and the inhibition of lipopolysaccharide (LPS) induced NO production in RAW264.7 macrophages by extracts of *M. subcordata* (MS) as well as selected candidate constituents thereof, identified by liquid chromatography coupled with multistage mass spectroscopy. U2OS-Cytotox cells were used in a counter assay that was performed to monitor cytotoxicity or any non-specific changes in luciferase activity. The results revealed that the fruit, root, and seed extracts were non-cytotoxic up to a concentration of 30 gram dry weight per litre (gDW/L) and induced PPARγ mediated gene expression but the leaf extract showed some cytotoxicity and exhibited minimal induction. Instead, all extracts showed concentration (1-15 gDW/L) dependent inhibition of LPS induced NO production. The root extract showed weaker inhibition. Among the candidate constituents, agmatine, stachydrine, trigonelline, indole-3-carboxyaldehyde, plus ethyl-, isobutyl-, isopropyl, and methyl-isothiocyanates showed similar inhibition, and most showed increased inhibition with increasing concentration (1-100 μM) although to a lesser potency than the positive control, aminoguanidine. In conclusion, the present study demonstrated for the first time the induction of PPARγ mediated gene expression by MS fruit, root, and seed extracts and the inhibition of LPS induced NO production by MS fruit, leaf, root, and seed extracts and some candidate constituents thereof. These vital properties of this plant may partly justify its traditional uses against infectious and metabolic syndrome related diseases.

Key words: peroxisome proliferator activated receptors gamma (PPARγ), extracts, gene expression, *Maerua subcordata*, nitric oxide

1. Introduction

The disease preventive and therapeutic potential of herbal medicines and foods rich in fruits, vegetables, and unrefined grains has been well established, although the detailed underlying mechanisms still need robust characterization [1-3]. Plant materials are composed of a vast array of bioactive principles, called phytochemicals, that are usually responsible for the beneficial or toxic outcomes of plant extracts [4]. Herbal medicines are characterized by a wider therapeutic indexes because they possess multicomponent mixtures of phytochemicals, which often have pleiotropic effects, targeting almost every molecular signaling pathway [5-6]. Therefore, the recognised disease preventive and therapeutic potential of botanicals has been linked to their phytochemical content that often produce a broad range of physiological effects [1,3].

Traditional medicines offer promising alternatives to biomedicine, especially with respect to therapies related to aging disorders, chronic and infectious diseases [7]. Because oxidative and inflammatory stresses are two most pivotal cellular stress response pathways that are linked to the pathogenesis of most major chronic diseases [1,8,9], modulation of these pathways by phytochemicals has been given a major focus as a possibly robust mechanism for the disease prevention and therapeutic potential of botanicals [10,11]. Another vital pathway modulated by phytochemicals is the peroxisome proliferator-activated receptors (PPARs) mediated signaling pathway. Many dietary phytochemicals are PPAR activators and ligand binding to PPARs has been proven to control several pathological conditions linked with obesity, aging-related diseases, inflammation, immune disorder, cell cycle regulation as well as cancer [12].

The PPARs are a subfamily of ligand activated transcription factors that belong to the nuclear receptor superfamily. In mammals, three major PPAR isoforms (α, β /δ, and γ) control the expression of diverse genes involved in metabolic homeostasis, adipogenesis, and inflammation [13-17]. PPAR dependent regulation of transcriptional activity is mediated by PPAR:retinoid X receptor (RXR) heterodimers [18]. Upon ligand activation, the PPAR/RXR heterodimers, which are bound to specific DNA sequence elements termed peroxisome proliferator response elements (PPREs) in the regulatory region of their target genes, recruit specific cofactors, thereby stimulating the transcription of target genes [18-20]. PPAR ligand binding leads to interactions with co-activators and/or co-repressors to induce or inhibit their functions. The function of PPARs is mainly regulated through ligand binding but also by some post-translational modifications such as phosphorylation, SUMOylation, ubiquitination, and acetylation which are found at numerous modification sites [21].

PPARs control lipid metabolism and inflammation [18,22]. Particularly, PPARγ activation is linked with beneficial health effects, including insulin-sensitization and immunomodulation with anti-inflammatory properties [20,23]. While all three PPAR isotypes demonstrate anti-inflammatory effects, PPARγ was the first for which the mechanism by which it inhibits inflammation was elucidated [18,24]. PPARγ undergoes ligand-dependent SUMOylation that results in its recruitment to the promoters of inflammatory genes where it inhibits transcription by stabilizing corepressor complexes. By this mechanism, PPARγ was shown to inhibit gene expression, in macrophages, of the nuclear factor kappaB (NF-κB) mediated inflammatory genes including the inducible nitric-oxide synthase (iNOS) gene [24,25]. Likewise, PPARγ agonists attenuate the induction of iNOS expression by lipopolysaccharide (LPS) [25].

The NF-κB family of transcription factors are master regulators of immune and inflammatory processes [26-28] that induce the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines [17]. For example, cytokines and gram negative bacterial endotoxins such as LPS were shown to cause NF-κB mediated induction of inducible nitric-oxide synthase (iNOS) gene expression and increased production of nitric oxide (NO) [29,30]. Activating PPARγ gene expression or inhibiting NF-κB pathways likely has a protective effect against inflammatory diseases [31] and PPARγ agonists were shown to demonstrate anti-inflammatory effects [32] and inhibition of iNOS [33] by interfering with the NF-kB signalling pathways [34].

Maerua subcordata (Gilg) DeWolf (Capparidaceae/Capparaceae) is a medicinal and (famine) food plant. Its root tuber and leaf parts are used in traditional medicine to treat infections and for wound healing [35-39]. Moreover, the root tuber is used to treat diabetes, high blood pressure, and allergic disorders as well as to improve appetite [40]. Inflammation is a common contributor to the pathology of all these disease conditions. It is now widely appreciated that low-grade chronic inflammation plays a key role in the initiation, propagation, and development of metabolic diseases, mainly in relation to obesity and type 2 diabetes, the metabolic syndrome, cancer, and cardiovascular diseases [26,41,42]. Although cure of inflammatory diseases is a significant challenge, medicinal herbs used in traditional medicine may signify a possible option for obtaining effective anti-inflammatory therapies [43]. Especially, Brassica vegetables are known for their preventive role against these inflammation related disorders, mainly due to their glucosinolate content. Glucosinolate hydrolysis products, the isothiocyanates, are known to play important roles in disease prevention by triggering antioxidant and anti-inflammatory responses, among others [44-47]. In our previous work, the nuclear factor (erythroid-derived

2)-like 2 (Nrf2) mediated antioxidant effect of *M. subcordata* different extracts and the presence, in these extracts, of phytochemicals such as glucosinolates was reported [48]. Thus, taking the above viewpoints into account, the present study is aimed to identify further endpoints that signify health benefits by assessing the potential of *M. subcordata* extracts and selected candidate constituents thereof, for induction of PPARγ mediated gene expression and inhibition of NF-kB/iNOS mediated NO production in LPS-activated RAW264.7 macrophages.

2. Materials and methods

2.1. Chemicals and reagents

Arecaidine hydrochloride was from Alfa Aesar (Karlsruhe, Germany); N-acetylagmatine from Cayman Chemicals-Europe (Sanbio Uden, The Netherlands); glucobrassicin potassium salt, stachydrine hydrochloride, and trigonelline hydrochloride from PhytoLab (Vestenbergsgreuth, Germany); agmatine sulfate, anthranilic acid, azeleic acid, bovine serum albumen (BSA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT), ethanol, indole-3-carboxaldehyde, isothiocyanates (methyl-, ethyl-, isobutyl-, isopropyl-isothiocyanates), geranylgeranylacetone, 9-hydroxyoctadecadienoic acid, α-linolenic, α-lipoic acid, lipopolysaccharide (from *Escherichia coli* 055:B5-γ-irradiated, BioXtra, suitable for cell culture), N-1-napthylethylenediamine dihydrochloride (NED), petroselinic acid, pipecolic acid, rosiglitazone, sclareol, stigmasterol, RRR-α-tocopherol, and Viscozyme L were from Sigma-Aldrich (Germany and The Netherlands), sulfanilamide (Sigma, China); ortho-phosphoric acid 85% and sodium nitrite were from Merck (Darmstadt, Germany). Minimum Essential Medium alpha 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12)(with and without phenol red), foetal calf serum (FCS), and Phosphate Buffered Saline (PBS) were from Gibco life technology (Paisley, UK); trypsin, nonessential amino acids (NEAA), and G418 were from Invitrogen Corporation (Breda, The Netherlands); and dextran-coated charcoal-stripped foetal calf serum (DCC-FCS) was purchased from Thermo Scientific (Waltham, USA).

2.2. Plant material

Different parts of *M. subcordata* (fruit, leaf, root tuber, and seed) were collected near Shiraro area (14.3970° N, 37.7743° E) of Northwest Tigray, Northern Ethiopia. Plant authentication was done in the National Herbarium at Addis Ababa University, Addis Ababa, Ethiopia where a specimen (Voucher number MG001/2007) was deposited. The plant parts were sorted and

dried in the laboratory of Pharmacognosy, Mekelle University, Mekelle Ethiopia. The root tuber was sliced into small pieces after its outer thin coating was peeled and the sliced pieces dried in an oven at a temperature of 40 ºC for four days. The other parts (fruit, leaf, and seed) were dried in the shade at room temperature and seeds were taken out of fruits after drying. The dried plant materials were packed in plastic bags, and stored at room temperature on shelf until they were transported to Wageningen University, The Netherlands; where they were powdered: each dried plant part was splashed with liquid nitrogen to remove moisture and facilitate powdering, and then powdered using an analytical miller. Each powdered plant material was mixed well, packed in capped plastic tubes, and stored at -80 ºC until further use.

2.3. Cell lines

Cytotox CALUX and PPARγ2 CALUX cells (Bio Detection Systems, Amsterdam, The Netherlands) as well as RAW264.7 murine macrophage-like cells (American Type Culture Collection) were used in the present study. The cytotox CALUX cells are human osteosarcoma U2OS cells stably transfected with a reporter construct carrying a luciferase reporter gene under transcriptional control of a constitutive promoter. They have an invariant luciferase expression and serve to determine cytotoxicity and to investigate whether stabilisation of the luciferase enzyme is occurring during chemical exposure [49,50]. The PPARγ2 CALUX cells are human osteosarcoma U2OS cells stably transfected with an expression vector for PPARγ2 and a reporter construct containing a luciferase gene under transcriptional control of the peroxisome proliferator responsive element [51,52]. Both PPARγ2 and Cytotox CALUX cells were cultured in DMEM/F12 glutamax supplemented with 7.5% FCS, and 1% NEAA. Once per week 200 µg/mL G418 was added to the culture medium in order to maintain selection pressure [51]. RAW264.7 cells are from a macrophage-like cell line derived from Balb/c mice. They maintain many of the properties of macrophages including nitric oxide production [53]. The RAW264.7 cells were grown and maintained in DMEM/F12 glutamax supplemented with 10% FCS, and 1% NEAA [54]. All cell lines were incubated at 37 ºC and 5% CO_2.

2.4. Preparation of extracts from *Maerua subcordata*

Enzyme (Viscozyme L) hydrolysed and non-hydrolysed methanol extracts from dried powders of *M. subcordata* fruit, leaf, root tuber, and seed samples were prepared following the procedure described by Gijsbers *et al*., (2012) [49] and Hiben *et al*., (2019) [48]. To prepare non-hydrolysed extracts, 3.4 ml methanol was added to 0.6 g plant material and vortexed. The mixture was sonicated (10 min), centrifuged (15 min, 1000g), the supernatant of each sample filtered using 0.2 µm polytetrafluoroethylene (PTFE)-filters (Whatman, Germany), and freeze-

dried after the methanol was evaporated under a stream of nitrogen. Dried extracts were stored at -80 ºC until further use. These extracts were re-dissolved in DMSO:DMEM (without phenol red)(1:2 v/v) [49] when preparing exposure medium for the assays with CALUX cell lines and in DMEM when preparing exposure medium for the assays with RAW264.7 cells. To prepare enzyme hydrolysed extracts, 300 µl sodium acetate (0.1 M, pH 4.8) and 100 µl of Viscozyme L were added to 0.6 g plant material, followed by 1 hr incubation in a water bath at 37 ºC. Then, samples were put on ice and 3.0 ml methanol was added to each sample, followed by 10 min sonication and 15 min centrifugation at 1000 g. The supernatant was then filtered, freeze-dried and stored at -80 ºC until further use similar to the procedure for the non-hydrolysed extracts. These enzyme-hydrolysed extracts were re-dissolved in DMSO: DMEM (without phenol red) (1:4 v/v) [49] while preparing exposure medium for the assays with CALUX cell lines.

2.5. CALUX assays

The PPARγ mediated gene expression induction potential of *M. subcordata* methanol extracts and selected candidate constituents was assessed by measuring induction of luciferase activity in PPARγ2 CALUX luciferase reporter cells. For each assay with PPARγ2 CALUX cells, an identical counter screen was done using the U2OS cytotox CALUX cells. Each plate included rosiglitazone (1 µM or 10 µM) as positive control and 1% (v/v) DMSO as a solvent control. In each well containing test samples, the final DMSO concentration was 1% (v/v). Assays were performed essentially as described by (Gijsbers *et al*., (2013) [51] and Beekmann *et al*., (2015) [52]. Briefly, the CALUX cells were seeded in the 60 inner wells of a white 96-well view plate at a density of $1x10^4$ cells per well in 100 µl assay medium consisting of DMEM/F12 without phenol red supplemented with 5% DCC-FCS, and 1% NEAA. The outer 36 wells were filled with 200 µl PBS to maintain physical homogeneity throughout the plate. While screening fatty acids, cells were seeded with assay medium containing 50 µM vitamin E (prepared by adding 20 µL of a 50 mM RRR-α-tocopherol solution to 20 mL of assay medium). The seeded cells were incubated for 24 hr to allow them to attach and form a confluent monolayer. The next day, 100 µl exposure medium containing the test samples was added to each well resulting in 200 µl assay medium per well. An assay medium containing 50 µM freshly added vitamin E and 0.1% BSA was used while preparing exposure medium containing fatty acids, and both positive and solvent controls. The vitamin E serves as an antioxidant to prevent oxidation of the unsaturated fatty acids while BSA facilitates the solubility and cellular availability of the fatty acids. After 24 hr exposure, medium was removed, cells were washed with ½ PBS (PBS half diluted with nano pure water) and then 30 µl low salt buffer was added to each well with cells.

The plates were subsequently frozen overnight at -80 °C in order to lyse the cells. Then plates were thawed and luciferase activity per well in the lysate was measured in relative light units (RLU) using a luminometer (GloMax-Multi Detection System-Promega, USA) upon adding 100 µl per well of flash mix. Results were described as fold induction compared to the solvent control. Extracts or compounds giving less than twofold induction at the highest concentration that could be tested without cytotoxicity were considered unable to induce PPARγ - mediated luciferase gene expression.

2.5.1. Alamarbleu (resazurin) assay

In addition to the cytotox reporter gene assay, the cytotoxicity of test samples was evaluated by the Alamarbleu (resazurin) assay. Cytotox CALUX and PPARγ2 CALUX cells were cultured in 96 well plates in their appropriate medium, described above, for 24 hrs and then the cells were exposed to test samples for another 24 hrs. Then alamarbleu (resazurin) reagent solution (10% w/v in PBS) was added directly to the cells (10% v/v i.e. 20 µl reagent to 200 µl cells in medium). Following reagent addition, plates were covered by aluminium foil, incubated for 2 hrs after which fluorescence was measured (λex 570/λem 585) using a plate reader (Molecular Devices, Spectra Max M2) equipped with Softmax Pro software.

2.6. Inhibition of nitric oxide production in RAW264.7 macrophages

The inducible nitric oxide synthase (iNOS) mediated nitric oxide production inhibition capacity of *M. subcordata* extracts and selected constituents was assessed using gram negative bacterial lipopolysaccharides (LPS)-stimulated RAW264.7 mouse macrophages. Assays were carried out essentially as described by Meijerink *et al*., (2011) [54]. Briefly, adherent RAW264.7 macrophage cells were scraped, suspended in fresh medium, diluted to a final density of $5x10^5$ cells/ml, and seeded by pipetting 100 µl ($5x10^4$ cells/well) of the cell suspension to the inner 60 wells of 96 well plates (transparent flat bottom). The outer wells were filled with 200 µl PBS and the plates were incubated for 24 hr. Stock solutions of each extract (in DMEM) and each candidate compound (in DMSO) were used to prepare exposure medium by serial dilution using medium alone and medium with LPS (400 ng/ml), each test concentration diluted 1000x. After careful aspiration of the culture medium, cells were exposed to the test samples by pipetting 100 µl of exposure medium containing each test concentration in triplicate. The DMSO final concentration was set to be 0.1% (v/v). Two solvent controls (medium only and medium with 0.1% (v/v) DMSO) were used, both with and without LPS. After 24 hr exposure, the supernatant medium in each well was transferred into a new plate to measure the nitrite level using the

Griess assay and 100 µl fresh medium were added to the remaining cells which were used for the MTT assay to assess cell viability.

2.6.1. The Griess assay

Assay principle: in biological systems, NO is rapidly oxidized by oxygen to nitrite (NO_2^-) and/or nitrate (NO_3^-), its two primary, stable and non-volatile breakdown products. The measurement of nitrate/nitrite concentration is routinely used as an index of NO production [55]. Measuring nitrite concentration, which relies on a diazotization reaction that was originally described by Griess in 1879, is a reliable method for quantifying NO production by cells [56]. Nitrite is first treated with a diazotizing reagent, sulfanilamide (SA), in acidic media to form a transient diazonium salt which is then allowed to react with a coupling reagent, N-1-naphthyl-ethylenediamine (NED), to form a stable azo compound. The intense purple colour of the product allows nitrite quantification at concentrations as low as ~0.5 µM. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample [55,57]. The present study followed this principle to measure the nitrite level, and thereby effects of test samples on NO production by cultured RAW264.7 macrophages. To this end, the supernatant of exposed cells in each well was transferred into a new plate. In empty rows of wells of the same plate, different concentrations (0 to 100 µM) of standard sodium nitrite were prepared in duplicate. Then 50 µl of 1%(w/v) SA in 5% phosphoric acid was added to all wells, and after 10 min incubation at room temperature, 50 µl 0.1%(w/v) NED dihydrochloride was added to all wells. Absorbance was measured at 540 nm using a plate reader (Molecular Devices, Spectra Max M2) within 10 to 30 min after reagent addition. NO production was estimated from the absorbance reading using an equation from a standard nitrite calibration curve. To show that induction of NO production by LPS was evident, exposures with and without LPS (LPS^+ and LPS^-, respectively) were related in each plate for each test concentration by comparing NO values of each test exposure (NO[t]) to NO value from LPS^+ medium control, representing maximum LPS-induced NO production (NO[M]). As the value of NO[M] may vary for different tests, and also to present results from different tests in one graph, data were further harmonized by dividing the mean NO[t] by the mean NO[M] value so that the NO[M] value for all tests represent 100%. %NO production = (mean NO[t]/mean NO[M])x100. For exposures with LPS, the %NO production values below 100% indicate inhibition of LPS induced NO production or otherwise cytotoxicity whereas values above 100%, if any, may indicate enhancement of LPS induced NO production by test samples. With the assumption that NO value from exposures to LPS^- medium control should represent background absorbance

(0% LPS-induced NO production) while NO value from exposures to LPS^+ medium control represent maximum measurement (100% LPS-induced NO production), percent inhibition of LPS-induced NO production by test samples was calculated from corrected NO values (background absorbance subtracted).

%Inhibition = {(meanNO[Mc]-mean NO[tc])/meanNO[M]}x100, where mean NO[Mc] is the mean value of corrected NO production from exposure to medium plus LPS and mean NO[tc]) is the mean value of corrected NO production from an exposure to each test concentration.

2.6.2. MTT Assay

The initial plates with the remaining cells were used in this assay. MTT reagent solution (5 mg/ml in PBS) was added directly to the cells (10% v/v i.e. 10 µl reagent/100 µl medium). Following reagent addition, plates were covered by aluminium foil, incubated for 1 hr, after that medium was carefully aspirated and formazan crystals were dissolved in 100% DMSO after which absorbance was measured at 570 nm using a plate reader (Molecular Devices, Spectra Max M2) equipped with Softmax Pro software.

2.7. LC-MS/MS based metabolic profiling of *M. subcordata*

Liquid chromatography (LC) coupled with multistage mass spectroscopy (MS) was applied to identify constituents in the methanol extracts from fruit, leaf, root, and seed of *M. subcordata* as described previously [48]. Briefly, methanol extracts of different plant parts were subjected to LC-MS analysis and generated spectral data were uploaded to 'Ms Annotation based on *in silico* Generated Metabolites' (MAGMa) for structural annotation. MAGMa is an application software (http://www.emetabolomics.org/magma) offered by Ridder *et al.*, (2012) [58] to help structural characterization of mixtures of metabolites present in complex extract samples. The MAGMa user interface displays alternative candidate structures, retrieved from Kegg, that are ranked on the basis of calculated matching score. The matching score helps to select candidate structures, from the user interface display, that are most probable constituents of the sample extracts. The identity of selected candidates was further confirmed and quantified by LC-MS/MS based on multiple reaction monitoring (MRM) and standard calibration curves. Optimum LC-MS/MS operating conditions were set using commercially available standard compounds. Varying concentrations of standard solutions, used to generate calibration curves, and filtered (0.2 µm polytetrafluoroethylene (PTFE)-filter) extract samples were analysed under identical conditions. The compounds of interest in the sample extracts were identified by their retention time and quantified based on calibration curves.

2.8. Data analysis

For each experiment, at least three independent tests were performed. Graphs present the average of repeated tests. Data were analysed using Microsoft excel 2016. For the CALUX assays, data are expressed as fold induction over the solvent control, and presented as mean values ± standard error of the mean (SEM). For assays with RAW264.7 macrophages, data are described as percent nitric oxide (NO) production or as percent inhibition of LPS induced NO production after subtracting the background absorbance from treatments without LPS. Each data point was measured, at least, in triplicate. Statistical significance was assessed using SPSS 23, paired samples statistics t-test. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Effect of *M. subcordata* methanol extracts on viability of U2OS CALUX cells

Results of the resazurin assay (**Fig 1a**) show that all the tested extracts up to a concentration of 30 gDW/L (gram dry weight per litre) were not cytotoxic (cell viability ≥80%) to both U2OS-Cytotox CALUX and U2OS-PPARγ CALUX cell lines. 30 gDW/L was used as screening concentration for the extracts in further reporter gene assays.

3.2. Induction of PPARγ mediated luciferase expression by *M. subcordata* extracts

Results of the U2OS-PPARγ CALUX assay (**Fig 1b**) show that both enzyme hydrolysed (eh) and non-hydrolysed (nh) extracts from the fruit, root, and seed materials as well as rosiglitazone (positive control) increased luciferase activity compared to 1% (v/v) DMSO as a solvent control implying induction of PPARγ mediated gene expression, whereas the induction by the leaf extracts was minimal. For all plant parts, the enzyme hydrolysed extracts showed slightly higher increase in luciferase activity than the matching non-enzyme hydrolysed extracts. To monitor non-specific interference with luciferase activity or cytotoxicity by the extracts, a parallel test was done using the U2OS-cytotox assay. Unlike the resazurin assay that showed non-cytotoxic effects for all the tested extracts, the cytotox assay showed that the leaf extracts reduced luciferase activity (69% and 47% remaining luciferase activity compared to the solvent control for eh and nh extracts, respectively) implying that the leaf extracts may have caused cytotoxicity or may have interfered with luciferase activity, ultimately resulting in minimal increase in luciferase activity. On the other hand, the fruit, root, and seed extracts showed similar activity as the solvent control indicating the absence of cytotoxicity in line with the resazurin assay. As luciferase activity at 30 gDW/L in the U2OS-cytotox assays were similar to that of the solvent control for these extracts, it was assumed that the extracts were non-cytotoxic and did not

interfere with luciferase itself. Thus, induction of PPARγ mediated gene expression by the fruit, root, and seed extracts observed in the U2OS-PPARγ CALUX assay was not caused by stabilization of the luciferase but reflected induction of gene expression.

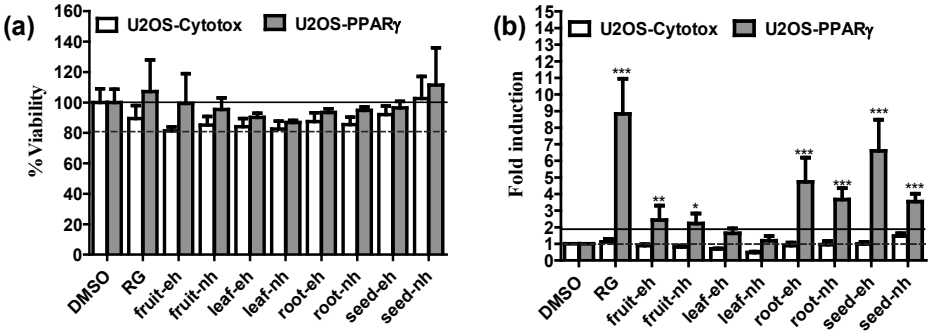


Figure 1. Effect on viability (a) and luciferase activity (b) of U2OS-Cytotox and U2OS-PPARγ cells after 24 hr exposure to rosiglitazone (RG,10 μM) and enzyme hydrolysed (eh) and non-hydrolysed (nh) methanol extracts of fruit, leaf, root and seed of *M. subcordata* tested at a concentration of 30 gDW/L. Cell viability (a) was measured by the resazurin assay with the viability of cells exposed to the solvent control (1% DMSO) set at 100%, and luciferase activity (b) was measured by the CALUX reporter gene assay and expressed as fold induction compared to solvent control. Data are presented as mean ± SEM of three independent experiments. Asterisks indicate a significant difference from the solvent control: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3. Some selected candidate compounds in *M. subcordata*

In an attempt to identify phytochemicals of *M. subcordata* with possible effect on the induction of PPARγ mediated gene expression and/or inhibition of inflammation pathways, LC-MS/MS analysis plus MAGMa software based structural annotation was done on the methanol extracts of each plant part that resulted in the tentative identification of different constituents from which candidates were selected based on literature reports (**Table 1**). Whereas the identification of constituents such as glucosinolates and some biogenic amines was reported in our previous work [48], the present report provides additional candidate constituents including guanidine derivatives (**S1a Fig**), quaternary ammonium compounds (betaines) (**S1b Fig**), and fatty acids and miscellaneous compounds (**S1c Fig**). Further LC-MS analysis using reference standard

compounds confirmed the presence of agmatine in all extracts and of indole-3-carboxyaldehyde (I3C) in fruit, leaf, and seed extracts of *M. subcordata* (**Table 2**). I3C is a breakdown product of glucobrassicin (GluB) (**Fig 2a**), an indolyl glucosinolate previously reported in *M. subcordata* with some aliphatic glucosinolates, stachydrine, and trigonelline [48]. The detection of I3C only in fruit, leaf, and seed extracts supports our previous report that GluB was detected only in these extracts. Besides, fatty acids with reported PPARγ agonist activity were part of the tentative identification but their definite identification was not accomplished in the present report and needs further work. Some selected candidates that were tested for their potential effect on induction of PPARγ mediated gene expression and/or inhibition of NO production in further experiments are presented in **Figure 2a-d**.

Table 1. Compounds tentatively identified as candidate constituents of *Maerua subcordata* and reported to be ligands of PPARγ and/or influence PPARγ functions

Compound	Literature
Agmatine	Agmatine increased gene expression of PPARα and PPARγ *in vivo* in rats [59].
Anthranilic acid	Anthranilic acid derivatives were reported as novel selective PPAR ligands [60,61].
Arecaidine	Arecaidine derivative, arecaidine methyl ester (arecoline) increased the translocation of glucose transporter type 4 *via* the PPARγ pathway [62].
Azelaic acid	Azelaic acid induced PPARγ mRNA and its transcriptional activity in human keratinocytes [63].
Indole-3-carboxaldehyde (derivative of indole-3-carbinol)	Indole-3-carbinol decreased expression of iNOS, decreased nitrite content and enhanced expression of PPARγ *in vitro* [64].
Isothiocyanates	Sulforaphane modulates NFκB and PPARγ signalling [65].
Geranylgeranylacetone (Teprenone)	Possible boosting of optimal PPARγ was suggested mechanism by which geranylgeranylacetone promote weight loss or improve insulin resistance in rodents and humans [66].
9-HODE	9-Hydroxyoctadecadienoic acid (9-HODE) is among the most proposed natural PPAR γ ligands [67].

α-Lipoic acid	α-Lipoic acid acts as a PPARγ agonist to counteract oxidative stress [68].
α-Linolenic acid	Polyunsaturated fatty acids including *α*-linolenic acid are natural ligands for PPARγ [69].
Petroselinic acid	Fatty acids including petroselinic acid and linolenic acid were identified as PPAR*γ* ligands [70].
Pipecolic acid (piperidine-2-carboxylic acid)	A pipecolic acid derivative stimulated transcriptional activities of PPARα and PPARγ and induced expression of their target genes [71].
Stachydrine	Stachydrine showed a PPARγ receptor glide score comparable to synthetic antidiabetic drugs [72].
Stigmasterol	Sterols like stigmasterol are known PPARγ agonists (can lower blood glucose) [73].
Trigonelline	Trigonelline increased insulin sensitivity and enhanced adipose tissue PPARγ activity in diabetic rats *in vivo* [74].

Table 2. Agmatine and indole-3-carboxyaldehyde in *M. subcordata* extracts identified and quantified by LC-MS/MS MRM and standard calibration curves. (a) Amount described in microgram per gram dry weight (µg/gDW) and (b) extrapolated concentration (µM) in 96 well plate as calculated from the maximum concentration (30 gDW/L) which was applied in the *in vitro* studies with plant extracts. Rt, retention time.

Samples	**Compounds**			
	Agmatine; Rt = 0.81 min		Indole-3-carboxyaldehyde; Rt = 9.51 min	
	a	b	a	b
Fruit	66.65	15.36	1.32	0.27
Leaf	24.51	5.65	0.84	0.17
Root	13.84	3.19	0.00	0.00
Seed	42.37	9.76	12.63	2.61

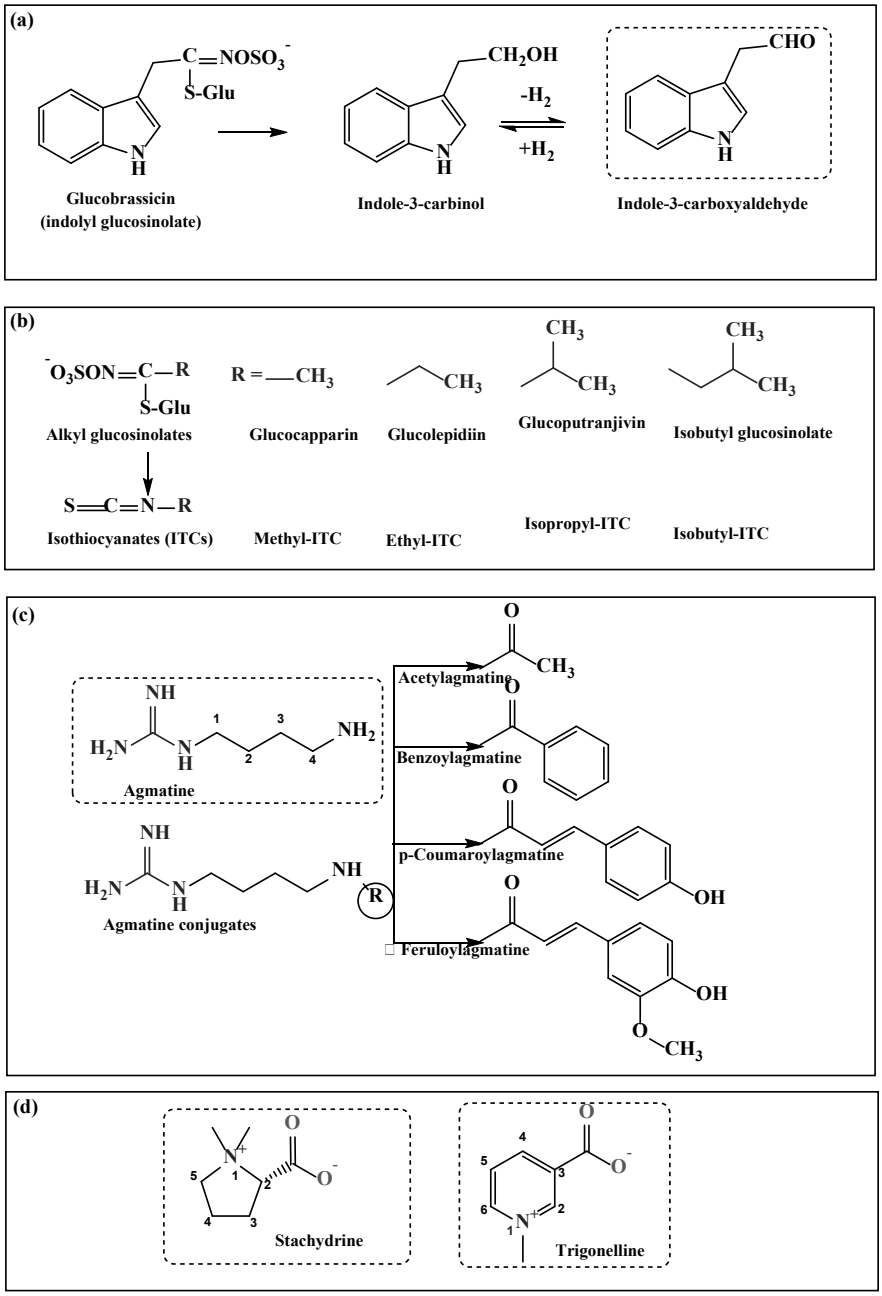


Figure 2. Representative phytochemical groups and constituents identified in *M. subcordata*. Boxes **a** and **b** show glucosinolates and their break down products, box **c** presents guanidines, and box **d** quaternary amines. Dotted boxes show candidates the presence of which was confirmed using standards.

3.4. Induction of PPARγ mediated luciferase expression by candidate constituents of *M. subcordata*

Different candidate constituents were screened for their PPARγ-mediated luciferase expression potential. The results (**Table 3**) revealed that α-lipoic acid and some candidate fatty acids such as α-linolenic acid, 9-hydroxyoctadecadienoic acid (9-HODE), and petroselinic acid showed biologically relevant (≥2 fold) induction of luciferase activity while the induction by the other screened candidates was minimal.

Table 3. Selected candidate constituents in *M. subcordata* screened for their potential induction of PPARγ-mediated luciferase expression.

Compounds	**Concentration (μM)**	**Fold induction (mean ± SEM**
(a) *Fold induction compared to 1%(v/v) DMSO as the solvent control*		
Agmatine sulfate	100	1.35±0.09
N-Acetylagmatine	100	1.17±0.30
Anthranilic acid	100	1.89±0.33
Arecaidine hydrochloride	100	0.95±0.03
Stachydrine hydrochloride	100	1.04±0.19
Trigonelline hydrochloride	100	1.09±0.11
Pipecolic acid	100	0.88±0.02
Indole-3-carboxaldehyde	100	1.51±0.11
Glucobrassicin potassium	10	1.05±0.08
Ethyl isothiocyanate	10	1.18±0.20
Isobutyl isothiocyanate	10	1.91±0.62
Isopropyl isothiocyanate	10	1.76±0.52
sec-Butyl isocyanate	10	1.27±0.14
Geranylgeranylacetone	25$^{\phi}$	1.0±60.06
Sclareol	5$^{\phi}$	1.15±0.12
α-Lipoic acid	250	**2.16±0.27***
(b) *Fold induction compared to 1%(v/v) ethanol as the solvent control*		
Rosiglitazone	1	**15.09±0.6****

9-Hydroxyoctadecadienoic acid	10[#]	**2.13±0.15***
α-Linolenic acid	100	**2.93±0.23***
Petroselinic acid	100	**2.22±0.53***
Azeleic acid	100	1.31±0.08
Stigmasterol	100	1.49±0.17

[ϕ]highest non-cytotoxic concentration, [#]amount of sample was not enough to test higher concentration, *significant difference from the solvent control: *$p < 0.05$; **$p < 0.01$.

3.5. Inhibition of LPS induced nitric oxide production in RAW264.7 macrophages by *M. subcordata*

As a measure of a possible anti-inflammatory effect, the potential inhibition of LPS induced nitric oxide (NO) production in RAW264.7 macrophages by *M. subcordata* methanol extracts and selected candidate constituents identified in these extracts were evaluated. LPS induced NO production was estimated using an equation from a standard calibration curve (**S2a Fig**). To check for any possible influence of the extracts on absorbance at 540 nm or if NO radical scavenging activity by the extracts may contribute to any change in absorbance, a test with and without addition of extracts (10 and 50 gDW/L final concentrations) to the standard nitrite (**S2b Fig**) was done which revealed no noticeable intrinsic influence by the extracts. **Figs 3a** and **4a** show results described as %NO production revealing that exposures without LPS (LPS^-) reflect background levels and the background NO value for all test samples is comparable to that of the medium control. This implies the test samples had no influence on the absorbance reading emanating from intrinsic property or induction of cytotoxicity. To further show that reduction in NO production by the tested samples was not due to cytotoxicity, the cell viability MTT assay was performed, which showed that the extracts (**Fig 3b**) as well as the tested compounds (**Fig 4b**) were non-cytotoxic at the tested concentrations. Finally, to show the net inhibition (free of background values) of NO production by the test samples, results (**Figs 3c** and **4c**) were described as percent inhibition of NO production. These results show that all *M. subcordata* (fruit, leaf, root, and seed) extracts revealed statistically significant ($p<0.05$) inhibition of LPS induced NO production (**Fig 3c**). Also, the tested candidate constituents and aminoguanidine, used as a positive control, inhibited LPS induced NO production (**Fig 4c)**.

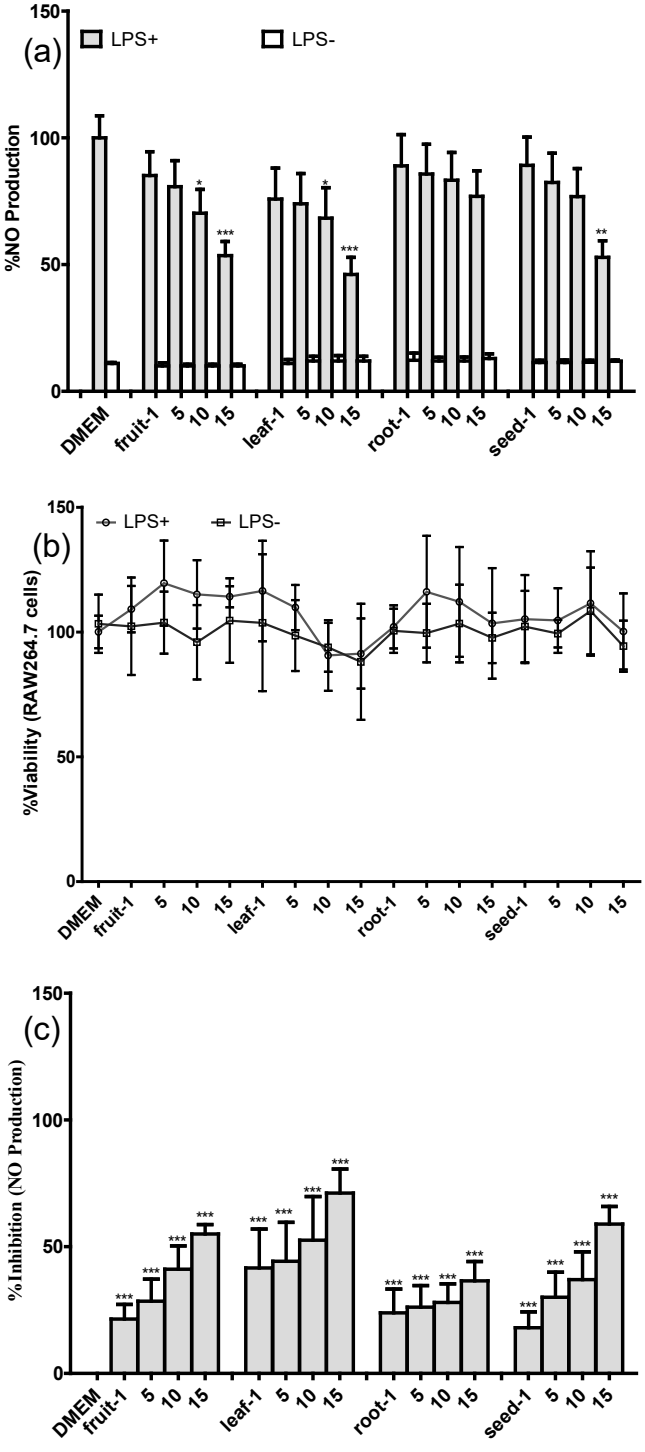

(a)
LPS+
LPS-
%NO Production
150
100
50
0
DMEM
fruit-1
5
10
15
leaf-1
root-1
seed-1
(b)
%Viability (RAW264.7 cells)
(c)
%Inhibition (NO Production)

Figure 3. Effect of *M. subcordata* extracts on NO production in LPS-induced RAW264.7 macrophages compared to medium control and showing the effect of the tested extracts on (**a**) NO production by cells with and without LPS (LPS^+ and LPS^-) treatments as measured by the *Griess assay*, **(b)** cell viability as measured by the *MTT assay*, and **(c)** description of %inhibition NO production. Data are presented as mean ± SEM and asterisks show a significant difference from control (medium+LPS): *p <0.05; **p <0.01; ***p < 0.001.

The results obtained (**Fig 4c**) reveal that many of the screened candidates show inhibition of LPS induced NO production, most of them in a concentration dependent manner, albeit to a weaker extent than the positive control, aminoguanidine. While stachydrine tended to show a concentration dependent decline in inhibitory activity, the activity by trigonelline seems to be independent of concentration, if not slightly decreasing with increasing concentration.

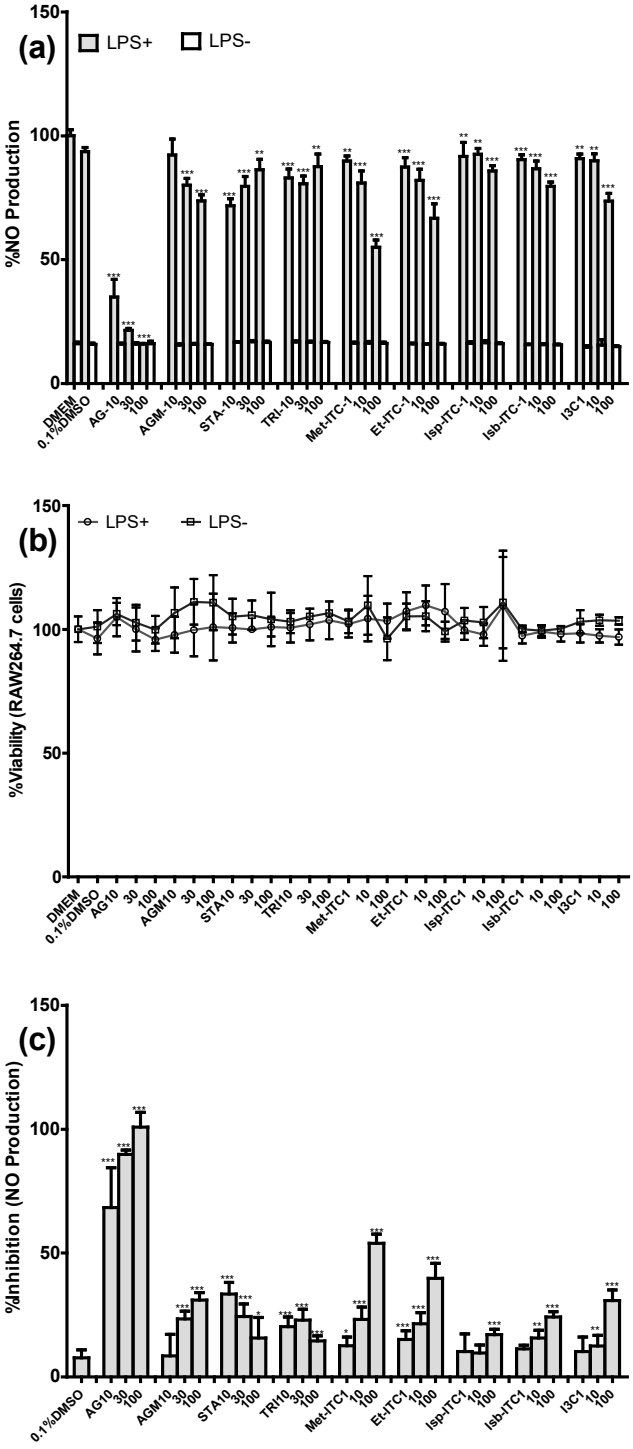

(a)
LPS+
LPS-
%NO Production
150
100
50
0
DMEM
0.1%DMSO
AG-10
30
100
AGM-10
STA-10
TRI-10
Met-ITC-1
10
Et-ITC-1
Isp-ITC-1
Isb-ITC-1
I3C1
(b)
%Viability (RAW264.7 cells)
AG10
AGM10
STA10
TRI10
Met-ITC1
Et-ITC1
Isp-ITC1
Isb-ITC1
(c)
%Inhibition (NO Production)

Figure 4. Effect of aminoguanidine (AG) as a positive control, agmatine (AGM), stachydrine (STA), trigonelline (TRI), indole-3-carboxaldehyde (I3C), and the isothiocyanates (ITCs): methyl- (Met-ITC), ethyl- (Et-ITC), isopropyl- (Isp-ITC), and isobutyl-isothiocyanate (Isb-ITC) on NO production in LPS-induced RAW264.7 macrophages compared to medium control and showing the effect of the tested candidates. (**a**) NO production by cells with and without LPS (LPS^+ and LPS^-) treatments as measured by the *Griess assay*, **(b)** cell viability as measured by the *MTT assay*, and **(c)** description of %inhibition NO production. Data are presented as mean ± SEM and asterisks show a significant difference from control (0.1%DMSO + medium + LPS): *p <0.05; **p <0.01; ***p < 0.001.

4. Discussion

The present study reports the induction of peroxisome proliferator activated receptor γ (PPARγ) mediated gene expression in U2OS-PPARγ cells and the inhibition of gram-negative bacterial lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW264.7 macrophages by *M. subcordata* extracts and candidate constituents thereof.

PPARs, a unique set of fatty acid regulated transcription factors that control lipid metabolism and inflammation [18,22], have emerged as therapeutic targets to treat various components of the metabolic syndrome [18]. In this regard, botanicals of dietary or medicinal products have attracted more and more attention owing to their relative effectiveness in upregulating PPAR signalling with fewer significant side effects [75-79]. Accordingly, *M. subcordata* fruit, root, and seed parts may provide possible health benefits by this mechanism as extracts from these plant parts were shown to exhibit PPARγ mediated induction of luciferase gene expression.

Metabolic syndrome is a cluster of risk factors often associated with obesity and characterized by macrophage infiltration and activation in adipose tissue and liver [18]. The nuclear factor kappaB (NF-κB) mediated induction of inducible nitric oxide synthase (iNOS) gene expression is up-regulated in tissues affected by inflammatory responses and in macrophages in response to inflammatory stimuli or pro-inflammatory cytokines that result in overproduction of NO [29,30,56,80,81]. Although NO is an important molecule, involved in regulation of many physiological and micro biocide processes, its overproduction is implicated in the pathogenesis of several chronic inflammatory and immunologically mediated diseases, and in complications of diabetes and obesity [56,82-84]. As NO is a key mediator of inflammation [83,84], inhibition

of inflammatory stimuli-induced NO accumulation has been suggested as a beneficial therapeutic strategy [85]. Natural products including medicinal plants provide a vast pool of NO inhibitors [86]. Likewise, extracts of *M. subcordata* (fruit, leaf, root, and seed) inhibited LPS-induced NO production in RAW264.7 macrophages to a varying extent, with the root extract showing weak inhibition. Since *M. subcordata* is used in traditional medicine to manage infectious and chronic diseases, and since inflammation is a common contributor to the pathology of these diseases, the inhibition of NO production by *M. subcordata* extracts may partly justify its traditional claims and also may show its potential as anti-inflammatory agent. Moreover, selected candidate constituents from this plant exhibited induction of PPARγ mediated gene expression or inhibition of NO production, which may substantiate its anti-inflammatory potential.

In line with the established evidence that endogenous or dietary fatty acids are known ligands of PPARs [87,88], the candidate fatty acids α-linolenic acid, 9-hydroxyoctadecadienoic acid (9-HODE), and petroselinic acid showed biologically relevant (≥2 fold)[89] induction of luciferase activity in the present work (**Table 3**). On the other hand, although an *in vivo* study in rats showed that agmatine increased the gene expression (levels of mRNA) of PPARα and PPARγ [59], both agmatine and its acetyl conjugate did not induce an increase in luciferase expression at the protein level in the present study. This may happen since a gene's mRNA level does not usually predict its protein level[90] due to unpredictable changes during translation. Stachydrine and trigonelline are constituents of medicinal plants used in traditional medicine for treatment of diabetes and the metabolic syndrome [72,91]. Both compounds showed a PPARγ receptor glide score (ligand binding free energy) comparable to synthetic antidiabetic drugs [72]. Yet, no activity above solvent control was detected for stachydrine in luciferase reporter gene assays of all three PPAR (α, β/δ, and γ) isoforms [91] while reports on trigonelline seem contradicting. Trigonelline increased insulin sensitivity and enhanced adipose tissue PPARγ activity in diabetic rats *in vivo* [74]. However, in *in vitro* tests, trigonelline showed either no effect [92] or down regulated [93] PPARγ gene expression in cultured cells. In line with these reports, both compounds showed no induction of PPARγ mediated luciferase activity above the solvent control in the present study. Overall, most tested candidates, except for some fatty acids, showed no biologically relevant induction of PPARγ mediated-luciferase expression (**Table 3**) and hence were considered inactive. The candidates that showed little or no PPAR*γ*-mediated luciferase activity, though reported as PPAR*γ* ligands and/or to impact PPAR*γ* functions, inhibited NO production, which may be justified by the fact that the mechanism by which

PPARγ and its agonists inhibit iNOS expression and NO production requires ligand-dependent PPARγ SUMOylation [24] and that the PPARγ gene is not vital for the inhibition of iNOS by PPARγ agonists [33]. This implies that these candidates may have minor impact on PPARγ gene but possible posttranscriptional regulation of PPARγ function.

Alternatively, most tested candidate constituents including agmatine, stachydrine, trigonelline and some break down products of glucosinolates like methyl-, ethyl-, isobutyl-, and isopropyl-isothiocyanates, and indole-3-carboxyaldyhyde were shown to inhibit NO production in LPS-stimulated RAW264.7 macrophages, albeit to an extent less potent than aminoguanidine, a selective iNOS inhibitor used as a positive control. Several plant derived natural products were shown to exert anti-inflammatory effects *via* inhibition of the NF-κB/iNOS pathway mediated NO production [46,94-97]. Therefore, the screened candidate constituents may act by this mechanism and may contribute to the NO production inhibition effect by the extracts.

Pharmacological modulation of iNOS activity has been achieved using structural analogs of arginine [98]. Agmatine (decarboxylated arginine), agmatine conjugates, and related guanidine derivatives (**S1a Fig**) were identified in *M. subcordata*. Reports support that aminoguanidine [82,99,100] and agmatine[101-103] are selective iNOS inhibitors. Both compounds were shown to attenuate the activation of NF-κB and its pro-inflammatory target genes [100,103]. Other guanidine analogs like guanidinosuccinic acid, guanidinoproprionic acid, methylguanidine [104], 1-amino-2-hydroxy-guanidine [105], and guanidinoethyldisulphide [106] were also shown to inhibit NO production implying that agmatine and its conjugates could have partly contributed to the inhibition of LPS-induced NO production by *M. subcordata* extracts.

Besides, the inhibition by the other constituents such as stachydrine, trigonelline, the candidate isothiocyanates, and indole-3-carboxyaldyhyde could have contributed to the inhibition of NO production by *M. subcordata* extracts, possibly providing additive or synergistic effect as the activity of the extracts seems to be stronger than that of the individual candidate constituents that were tested. Indeed, adequate literature support exists on stachydrine, trigonelline, and the glucosinolate breakdown products. *In vitro* and *in vivo* studies show that stachydrine exhibits inhibition of the NF-κB signalling pathway, which was suggested as a possible mechanism for its cardioprotective and anti-hypertrophic [107-109], hepatoprotective [110], reduction of traumatic brain injury [111], reduction of cerebral impairment from ischemia re-perfusion [112], anti-endotoxin [113], and pilocytic astrocytoma suppression [47] effects. Likewise,

trigonelline showed antidiabetic effects and counteracted insulin resistance by suppressing inflammation [47,109,114]. Also, ITCs have been linked with lower cancer risk [115,116] *via* suppressing NF-κB signalling pathways that lead to attenuated pro-inflammatory mediators and activities. ITCs were shown to reduce several pro-inflammatory mediators and cytokines, including iNOS, apparently by downregulation of NF-κB signalling pathways [115,117]. To mention some, 6-(methylsulfinyl) hexyl- [118], β-phenylethyl-, 8-methylsulphinyloctyl- [119], benzyl- [120], phenethyl- [121], and allyl- [46,122] isothiocyanates as well as indole-3-carbinol [123] were demonstrated to inhibit LPS-induced NO production in RAW 264.7 macrophages.

Taken together, the present study verified the induction of PPARγ mediated gene expression and the inhibition of LPS induced NO production by *M. subcordata* extracts and candidate constituents thereof. Different plant parts contain active compounds that act at multiple targets in the inflammatory response pathways and regulate a multitude of chemical mediators, enzymes, genes or cellular functions to alleviate inflammation [43] and hence, outcomes of assays that utilize extracts are expected to reflect a net effect of such potential multi-target interactions that may also apply to *M. subcordata* extracts. The PPARγ agonists' ability to inhibit inflammatory responses by repressing NF-κB target genes has been linked to the prevention and treatment of the metabolic syndrome and diabetes [124,125]. Likewise, the induction of PPARγ mediated gene expression as well as inhibition of NO production by *M. subcordata* extracts and candidate constituents thereof may imply that *M. subcordata* may have anti-inflammatory effects possibly by inhibiting NF-κB signalling pathways, which in turn may partly justify its use in traditional medicine to manage infectious and chronic diseases associated to the metabolic syndrome.

5. Conclusion

The present work showed for the first time the induction of PPARγ mediated gene expression by *M. subcordata* fruit, root, and seed extracts and the inhibition of LPS induced NO production in RAW264.7 macrophages by the fruit, leaf, root, and seed extracts and candidate constituents thereof that included agmatine, stachydrine, trigonelline, and glucosinolate breakdown products. As LPS induced NO production is mediated by the NF-κB/iNOS pathway and since PPAR-γ mediated gene expression or inhibition of the NF-κB pathway likely have a protective effect against inflammatory diseases, the observed effects may in part be in line with the traditional use of *M. subcordata* against infectious and chronic diseases.

Conflicts of Interest

The authors declare no conflict of interest.

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Appendix: Supporting Information

Supporting information can be found at the end of this chapter

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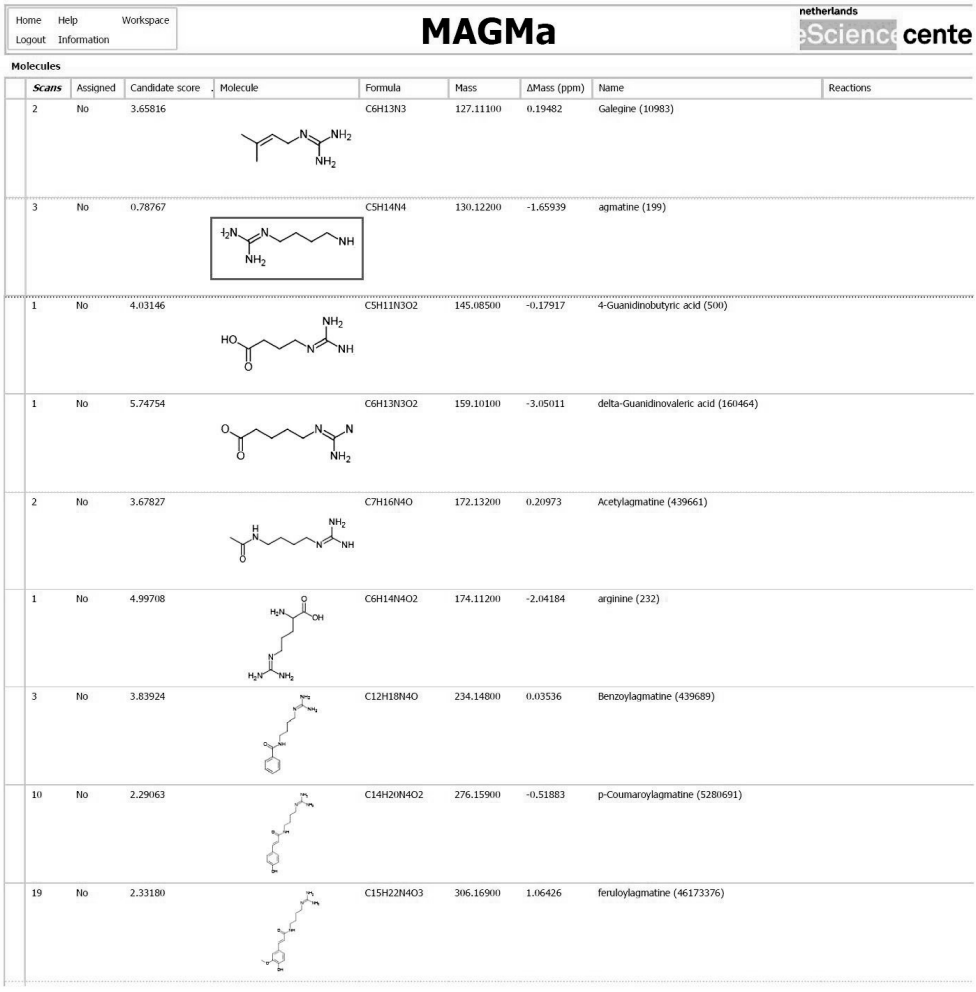
Appendix: Supporting Information

S1 Fig. Some candidate constituents tentatively identified in *M. subcordata* methanol extracts as displayed on MAGMa interface. (**a**) guanidine derivatives, (**b**) quaternary ammonium compounds (betaines), and (**c**) fatty acids and miscellaneous compounds.



a

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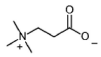
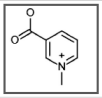
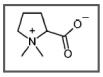
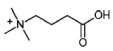
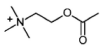
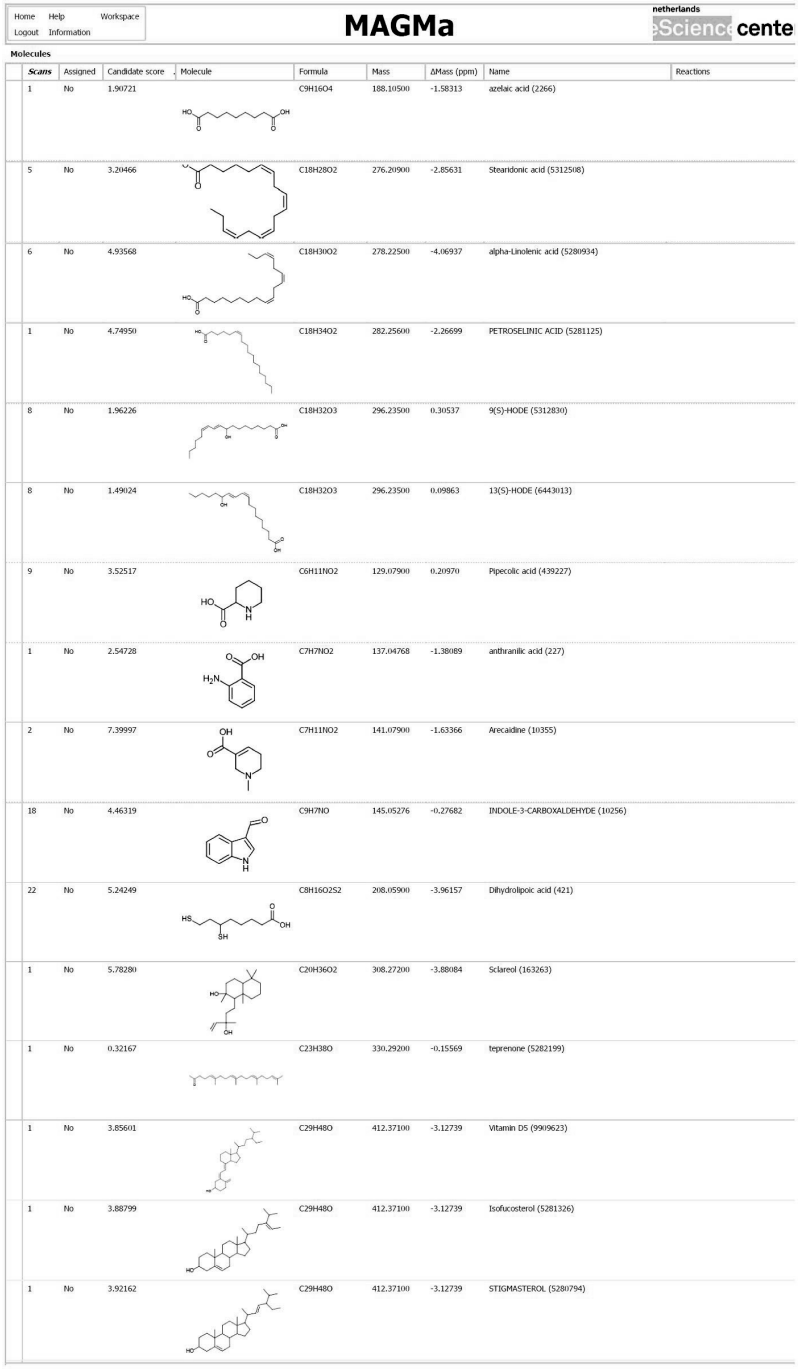
Molecules

Scans	Assigned	Candidate score	Molecule	Formula	Mass	ΔMass (ppm)	Name	Reactions
2	No	4.09491		C5H11NO2	117.07900	1.58779	betaine (247)	
3	No	0.93510		C6H13NO2	131.09500	-2.74773	Propiobetaine (441440)	
1	No	1.04094		C7H7NO2	137.04800	-2.82908	Trigonelline (5570)	
27	No	4.89969		C7H13NO2	143.09500	-3.04836	stachydrine (115244)	
1	No	3.13020		C7H16NO2+	146.11800	-0.03080	gamma-butyrobetaine (134)	
1	No	3.86928		C7H16NO2+	146.11800	-0.03080	acetylcholine (187)	
3	No	8.25592		C8H15NO2	157.11000	1.85013	Homostachydrine (4479243)	
22	No	1.97166		C7H13NO3	159.08954	-0.77757	3-Hydroxystachydrine (46173781)	

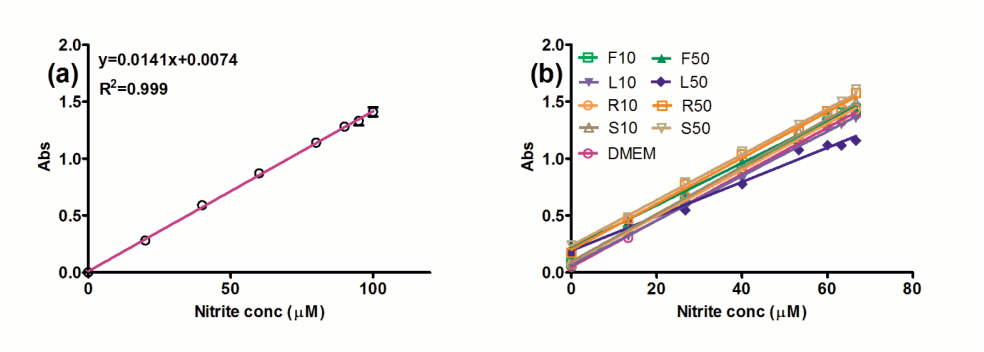
b



Scans	Assigned	Candidate score	Molecule	Formula	Mass	ΔMass (ppm)	Name	Reactions
1	No	1.90721		C9H16O4	188.10500	-1.58313	azelaic acid (2266)	
5	No	3.20466		C18H28O2	276.20900	-2.85631	Stearidonic acid (5312508)	
6	No	4.93568		C18H30O2	278.22500	-4.06937	alpha-Linolenic acid (5280934)	
1	No	4.74950		C18H34O2	282.25600	-2.26699	PETROSELINIC ACID (5281125)	
8	No	1.96226		C18H32O3	296.23500	0.30537	9(S)-HODE (5312830)	
8	No	1.49024		C18H32O3	296.23500	0.09863	13(S)-HODE (6443013)	
9	No	3.52517		C6H11NO2	129.07900	0.20970	Pipecolic acid (439227)	
1	No	2.54728		C7H7NO2	137.04768	-1.38089	anthranilic acid (227)	
2	No	7.39997		C7H11NO2	141.07900	-1.63366	Arecaidine (10355)	
18	No	4.46319		C9H7NO	145.05276	-0.27682	INDOLE-3-CARBOXALDEHYDE (10256)	
22	No	5.24249		C8H16O2S2	208.05900	-3.96157	Dihydrolipoic acid (421)	
1	No	5.78280		C20H36O2	308.27200	-3.88084	Sclareol (163263)	
1	No	0.32167		C23H38O	330.29200	-0.15569	teprenone (5282199)	
1	No	3.85601		C29H48O	412.37100	-3.12739	Vitamin D5 (9919623)	
1	No	3.88799		C29H48O	412.37100	-3.12739	Isofucosterol (5281326)	
1	No	3.92162		C29H48O	412.37100	-3.12739	STIGMASTEROL (5281794)	

c

S2 Fig. Calibration curves. (**a**) standard calibration curve used to estimate LPS induced nitric oxide production, (**b**) standard calibration curves with and without addition of extracts to the standard nitrite in order to check for false negative/positive results due to possible intrinsic influence of the extracts on the absorbance at 540 nm, the wavelength used to measure levels of nitric oxide.



Chapter 5

Hazard Assessment of *Maerua subcordata* (Gilg) DeWolf. for Selected Endpoints using a Battery of *In Vitro* Tests

Mebrahtom Gebrelibanos Hiben, Lenny Kamelia, Laura de Haan, Bert Spenkelink, Sebastiaan Wesseling, Jacques Vervoort, Ivonne M.C.M. Rietjens

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Abstract

Ethnopharmacological Relevance: *Maerua subcordata* (Gilg) DeWolf is a medicinal and wild food plant growing mainly in east Africa. Especially its root tuber is widely used in traditional medicine to treat several infectious and chronic diseases but also in some toxicity implications like use as abortifacient. *Aim of the study*: the present study applied *in silico* and *in vitro* tests to identify possible hazards of *M. subcordata* (fruit, leaf, root, seed) methanol extracts focusing on developmental toxicity. *Materials and methods*: Ames test, estrogen receptor alpha (ERα) assay, aryl hydrocarbon receptor (AhR) assay, embryonic stem cell test (EST), and zebrafish embryotoxicity test (ZET) were employed. Besides, a Derek Nexus toxicity prediction was performed on candidate structures obtained from metabolomics profiling of the extracts using liquid chromatography coupled to multistage mass spectroscopy (LC/MS^n) and a MAGMa software based structural annotation. *Results*: Glucosinolates, which degrade to isothiocyanates, and biogenic amines were among the candidate molecules identified in the extracts by LC/MS^n - MAGMa software structural annotation. Isothiocyanates and some other candidate molecules suggested a positive mutagenicity alert in Derek toxicity predictions. All the extracts showed negative mutagenicity in the Ames test. However, the Derek predictions also identified endocrine and developmental toxicity as possible endpoints of concern. This was further assessed using *in vitro* tests. Results obtained reveal that leaf extract shows AhR and ERα agonist activities, inhibited differentiation of ES-D3 stem cells into contracting cardiomyocytes in the EST ($p<0.001$) as well as inhibited hatching ($p<0.01$) and showed acute toxicity ($p<0.01$) in the ZET. Also, the fruit extract showed toxicity ($p<0.05$) towards zebrafish embryos and both fruit and seed extracts showed AhR agonist activities while root extract was devoid of activity in all *in vitro* assays. *Conclusion*: The leaf extract tests positive in *in vitro* tests that may point towards a developmental toxicity hazard. The current evaluations did not raise concerns of genotoxicity or developmental toxicity for the fruit, seed and root extracts. This is important given the use of especially these parts of *M. subcordata*, in traditional medicine and/or as (famine) food.

Key words: CALUX assays, *in vitro, Maerua subcordata,* hazard, embryonic stem cell test, zebrafish embryotoxicity test

1. Introduction

Human exposure to natural ingredients of botanical origin, particularly *via* the use of dietary supplements and herbal medicines, continues to rise globally[1,2]. The general misconception that "natural" always means "safe" together with the improved scientific data and understanding of the beneficial health effects of bioactive substances in plant foods and medicinal products have boosted worldwide use of botanicals [3-9]. Thus, both consumers and industry have a rising interest towards the development of food products, having 'functional' properties or health benefits, derived from botanicals ranging from staple food sources like cereals, fruits and vegetables, to herbals used in traditional medicine [5,10]. However, users may also get exposed to possible health hazards arising from inherent constituents or contaminants of botanical products that may lead to adverse health effects [6,10] and hence, there is a need to evaluate their hazards and safety.

Toxicity studies of botanicals are challenging and complicated due to, among others, costs, time, and animal usage as well as the complex mixtures of constituents that show variability in composition [4,11]. Still, an integrative testing strategy has been suggested tailored to hazard assessment using a panel of available alternative tests for critical end points that provides optimal *in silico* and *in vitro* filters [12] aimed to assess hazards of botanical products especially when safety data are insufficient and as an early screen for toxicity alerts, particularly for less commonly known botanicals [2]. Some suggested alternative methods include an *in silico* decision tree approach [4,11], a battery of *in vitro* tests such as assays for genotoxicity, CALUX reporter gene assays, the embryonic stem cell test (EST), and the zebrafish embryotoxicity test (ZET) [13-18].

A long history of use in traditional medicine of a botanical material is generally assumed as an indicator of lack of obvious toxicity [19]. Yet, safety issues need scientific justification if use of a botanical material is to be considered. *Maerua subcordata* (Gilg) DeWolf (Capparidaceae), a medicinal and famine food plant, has numerous traditional uses but it lacks scientific data on its potential health hazards and health benefits. Its tuber is claimed as safe antimalarial remedy for children and pregnant women in northern Ethiopia, where collection of plant material in the present study was made, while reports from Somalia show that the same plant part is used as abortifacient [19,20], which, if the later claim is true, may point at a possible adverse effect. Moreover, few reports reflect that *M. subcordata* could be potentially toxic to humans and animals. It has been marked as a noxious shrub, which is potentially toxic to wildlife and cattle

[21]. Human safety concern could be that uncooked fruits are perceived as toxic unless well boiled to render them to be non-toxic and edible [22,23]. Also, possible intoxication by quaternary ammonium compounds in the root was suspected, although such a problem was not detected along the area of the Omo river where the root is commonly used as a water clarifying agent; maybe because the quantities ending up in the water were too small to cause negative health effects [25]. Scientific data on *M. subcordata* are lacking. A study with brine shrimp (*Artemia salina* Leach) showed that a methanolic root extract was inactive (LC_{50} > 1000 µg/ml) against the tested organism [26] which could be an indication of absence of obvious toxicity, at least to this species. Therefore, considering these ethno-toxicological claims, the aim of the present study was to investigate the possible hazards related to use of parts or extracts of *M. subcordata* given that various ethno-medicinal claims on *M. subcordata* may point towards its potential uses as source of herbal medicine or as functional or famine food. A Derek Nexus toxicity prediction, performed on candidate structures obtained from metabolomics profiling of the extracts, was used to direct selection of the *in vitro* tests to be employed. Different *in vitro* tests were applied to assess especially the genotoxicity, and endocrine and developmental toxicity hazards of *M. subcordata* (fruit, leaf, root, and seed) methanol extracts.

2. Materials and Methods

2.1. Plant material: collection, authentication, and processing

The fruit, leaf, root tuber, and seed parts of *M. subcordata* (Voucher number MG001/2007) were obtained from plants collected at 'lemlem' sub-district near shiraro (14.3970° N, 37.7743° E) in Northwest Tigray, Northern Ethiopia. The fruit, leaf, and seed parts were dried at room temperature while the tuber was first chopped into small pieces and dried in an oven at 40 ºC. The dried plant materials were packed in plastic bags, transported to the Division of Toxicology, Wageningen University & Research, the Netherlands; where they were further processed and tested. To remove moisture and facilitate powdering, each dried plant part was splashed with liquid nitrogen and then ground using an analytical electric mill, mixed well, packed in capped plastic tubes, and stored at -80 ºC until further use.

2.2. Cell lines

ERα CALUX®, Cytotox CALUX®, and DR CALUX® cell lines were obtained from Bio Detection Systems BV (Amsterdam, The Netherlands). The pluripotent mouse ES-D3 cell line used for the EST was obtained from ATCC (ATCC® CRL 1934™, Wesel, Germany). The ERα

CALUX cells are human bone osteosarcoma U2OS cells stably transfected with a firefly luciferase gene coupled to estrogen responsive elements (EREs) as a reporter gene to detect estrogen receptor agonists and antagonists [14]. The Cytotox CALUX cells are human osteosarcoma U2OS cells stably transfected with a reporter construct carrying a luciferase reporter gene under transcriptional control of a constitutive promoter. These cells have an invariant luciferase expression and were originally designed to study cytotoxicity [27]. The ERα CALUX and Cytotox CALUX cells were cultured in Minimum Essential Medium alpha 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) (Gibco life technology™, Paisley, UK), supplemented with 7.5% foetal calf serum (FCS) (Gibco life technology™, Paisley, UK), and 1% nonessential amino acids (NEAA) (Invitrogen Corporation Breda, The Netherlands). The DR-CALUX cells are cells from a rat hepatoma (H4IIE) cell line, stably transfected with a construct containing the dioxin-responsive element sequence coupled to an AhR regulated luciferase reporter gene (H4I1E-*luc*) [28,29]. The DR-CALUX cells were cultured in Minimum Essential Medium alpha (α-MEM)) (Gibco life technology™, Paisley, UK) supplemented with 10% FCS [29,30]. The U2OS ERα, U2OS Cytotox, and H4IIE.luc (DR CALUX) cells were incubated at 37ºC with 5% CO_2 in a humidified atmosphere and subcultured every 2–3 days, using 0.05% trypsin-EDTA to detach the cells. The ES-D3 cell line used for the EST was maintained in 25 cm^2 polystyrene cell culture flasks (Corning, the Netherlands), pre-coated with 0.1% gelatine, in HyClone AdvanceSTEM™ Low Osmo Dulbecco's Modified Eagle Medium (DMEM) (Fischer Scientific, Landsmeer, the Netherlands) supplemented with 15% Fetal Bovine Serum (FBS) (ATCC, USA), 2 mM L-glutamine (Invitrogen, The Netherlands), 50 U/ml penicillin (Invitrogen) and 50 µg/ml streptomycin (Invitrogen). Cells were routinely subcultured every 2–3 days using non-enzymatic cell dissociation solution (Sigma-Aldrich, Schnelldorf, Germany) to detach the cells and were kept undifferentiated by the addition of 1000 U/ml murine Leukemia Inhibiting Factor (mLIF) (Sigma-Aldrich) [31]. The ES-D3 cells were incubated at 37 ºC and 5% CO_2 in a humidified atmosphere.

2.3. Zebrafish eggs

Eggs of zebrafish (*Danio rerio*) wild-type AB line, produced by group spawning were obtained from the Animal Sciences Group of Wageningen University & Research; Carus-ARF Bornse Weilanden 5, 6708 WG Wageningen, the Netherlands.

2.4. Preparation of extracts from *Maerua subcordata*

Methanol extracts from dried powders of different parts - fruit, leaf, root tuber, and seed- of *M. subcordata* were prepared by adding 3.4 ml methanol to 0.6 g of powdered plant material followed by sonication for 10 min and centrifugation at 1000g for 15 min. The supernatant was filtered using 0.2 µm polytetrafluoroethylene (PTFE)-filters (Whatman™, Germany) and freeze-dried after the methanol was evaporated under a stream of nitrogen. Dried extracts were stored at -80 ºC until used. For screening in the ERα CALUX and Cytotox CALUX assays, the extracts were re-dissolved in DMSO:DMEM (1:2 v/v) while for screening in the DR-CALUX, EST, Ames test, and ZET, the extracts were re-dissolved in DMSO (Sigma–Aldrich).

2.5. Ames Test

The plate incorporation method of the Ames test was used to screen the mutagenicity of *M. subcordata* fruit, leaf, root, and seed extracts at concentrations of 15, 30, and 60 milligram dry weight (mgDW) per plate. The extracts were tested with and without metabolic activation as described by Ames *et al*., (1975) [32] and with adaptions of the principles of the OECD 471 guideline [33]. Briefly, 0.1 ml test extracts or 0.25% (v/v) DMSO (solvent control), 0.1 ml overnight bacterial (*Salmonella typhimurium,* TA98 or TA100) culture (1 x 10^8 cells/ml), and 0.3 ml S9 mixture (11-403L MUTAZYME 5% S9 from Aroclor 1254-induced male SD rat liver, reconstituted with 20 ml cold sterile water) or PBS (for tests without metabolic activation) were added into 2 ml top agar in test tubes. The contents were mixed and poured onto the surface of a glucose minimal plate. After solidification, plates were incubated upside down at 37 °C for 72 hr. At the end of the incubation, revertant colonies were counted. For each test, an average revertant colony count of three independent experiment was presented. A test plate was considered positive for mutagenicity if it produced a number of revertant colonies significantly higher (>twofold) than spontaneous revertant colonies on the solvent control plate, or otherwise considered a negative result.

2.6. Metabolomics profiling and toxicity prediction

M. subcordata methanol extracts (5 µl) were injected onto a C18 column (Phenomenex Luna 150 x 2 mm i.d., 3 □m Torrance, USA) of an Ultimate 3000 UPLC with a 60 minutes gradient and measured on a Q Exactive Orbitrap FTMS System (Thermo Scientific, Bremen) in both positive and negative mode. The raw data were converted into mzXML by the ProteoWizard MSconvert software. Structural characterization was supported by 'MS Annotation based on in silico Generated Metabolites' (MAGMa) (http://www.emetabolomics.org/magma) software [34]. Uploaded spectral data were automatically annotated with tiered trees of *in silico*

generated substructures of candidates, retrieved from a subset of compounds present in Kegg. Candidate structures thought to be most likely present in the extracts were manually selected from the MAGMa user interface. The identity of selected candidates was further confirmed by LC-MS/MS (Shimadzu LC-MS 8040) based on multiple reaction monitoring (MRM) using market available standard compounds such as stachydrine hydrochloride, trigonelline hydrochloride (PhytoLab,Vestenbergsgreuth,Germany), glucolepidiin potassium, glucobrassicin potassium (Extrasynthese,Genay Cedex,France). Likewise, although structures of pyrrolizidine alkaloids (PAs) such as senecionine or senecivernine were in the tentative identification, their presence in the extracts was ruled out by further LC-MS/MS analysis using different standard mixture of PAs and with ≤ 1 µg/g limit of detection.

Toxicity prediction for query structures was performed using Derek Nexus, a software program developed by Lhasa Ltd. (https://www.lhasalimited.org/) [35]. In Derek, toxicity predictions are the result of two processes: (i) the program checks whether any alerts in its knowledge base match toxicophores (substructures known or thought to be responsible for a particular effect or toxicity) in the query structure and (ii) the reasoning engine assesses the likelihood of a structure being toxic described by nine confidence levels that were converted into three categories: active (certain, probable, plausible), equivocal, and not active (doubted, improbable, impossible, open, and contradicted) [35].

2.7. Cell culture assays

2.7.1. DR CALUX assay (AhR assay)

The AhR agonistic activities of *M. subcordata* methanol extracts were measured by the DR CALUX assay [28,29]. H4IIE-*luc* cells were seeded in the 60 inner wells of a white 96-well view plate at a density of $3x10^4$ cells per well in 100 µL culture medium while 200 µl PBS was added to the outer 36 wells. After 24 hr incubation, the cells were inspected under a microscope and then exposed to 30 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Sigma–Aldrich), as positive control, 0.5%(v/v) DMSO as solvent control, and varying concentrations (0.024-12 gDW/L) of extracts in 100 µl exposure medium in the same plate. The final DMSO concentration in the incubations was 0.5% (v/v). After 6 hr or 24 hr exposure, medium was removed, cells were washed with ½ PBS (PBS half diluted with nano pure water), and exposed to 30 µl low salt lysing buffer (1.212 g Tris, 0.084 g dithiothreitol, 0.73 g 1,2-cyclohexylenedinitrilotetraacetic acid in a litre of nano pure water, pH 7.8) was added to each well to lyse the cells. Then, plates were covered with aluminium foil, placed on ice for 15 minutes and frozen overnight at -80 ºC. For the luminescence measurement, plates were thawed

at room temperature and luciferase activity per well in the lysate was measured in relative light units (RLU) using a luminometer (GloMax®-Multi Detection System–Promega) after the addition to each well of 100 µl flash mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4.7H_2O$, 0.1 mM EDTA, 2.0 mM dithiothreitol, 470 µM luciferine, 5.0 mM ATP; in a litre of nano pure water, pH 7.8). At least three independent experiments were conducted and results were expressed as fold induction of luciferase activity compared to the solvent control.

2.7.2. ERα CALUX and Cytotox CALUX assays

The cytotox CALUX assay was done in the same way and parallel to every ERα CALUX assay because Cytotox CALUX cells serve as indicators of cytotoxicity or non-specific increases in luciferase activity. In brief, cells were seeded in the 60 inner wells of a white 96-well view plate at a density of $1x10^4$ cells per well in 100 µl assay medium: DMEM/F12 supplemented with 7.5% FCS, and 1% NEAA. The outer wells were filled with 200 µl PBS and the plates were incubated for 24 hr. The next day, the assay medium was carefully removed from the wells with cells, and replaced by 100 µl of fresh assay medium. The plates were incubated for another 24 hr. Then 100 µl exposure medium containing the test samples was added to each well resulting in 200 µl per well assay medium. After 24 hr exposure, medium was removed, cells were washed with ½ PBS, lysed with low salt buffer, frozen overnight and luciferase activity was measured in the same way as described above for the AhR CALUX assay.

2.7.3. ES-D3 cell viability and differentiation assays

ES-D3 cell viability assay

The cytotoxicity of the test extracts to ES-D3 cells was assessed by the WST-1 assay which measures the extent of conversion of tetrazolium salts into a formazan dye by mitochondrial enzymes in metabolically active cells. The ES-D3 cell viability assay was done as previously described by Kamelia *et al*., (2017)[31]. Briefly, ES-D3 cells were seeded in 96-well plates (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) at a density of 2×10^5 cells/ml (one day exposure) or 10^4 cells/ml (five days exposure) in 100 µl medium (without mLIF) and incubated for one day to facilitate cell adherence. Then, cells were exposed to 100 µl of medium with or without test extracts (3 replicates/concentration) and incubated for one day or five days at 37 °C and 5% CO_2. All extracts were tested at a range of concentrations up to 1500 mgDW/L. The final concentration of DMSO was kept at 0.25% (v/v). After the incubation period, 20 µl of WST-1 reagent (Roche Diagnostics, Mannheim, Germany) was added to each well and cells were incubated for 3 h at 37 °C and 5% CO_2. Subsequently, the absorbance of the formed

formazan was measured at 440 nm using a SpectraMax M2 (Molecular Devices, Sunnyvale, USA). Cell viability was expressed as percentage of cell viability compared to the solvent control which was set at 100%. Wells containing culture medium without cells plus WST-1 reagent were used as a blank (background control) and used to define 0% viability. At least three independent experiments were done for each test extract, and results were expressed as % viability compared to the solvent control.

2.8. ES-D3 cell differentiation assay

The inhibitory potential of test extracts on differentiation of ES-D3 cells into contracting cardiomyocytes was assessed using the ES-D3 cell differentiation assay of the embryonic stem cell test (EST) as described by Kamelia *et al*. (2017) [31]. The first step of the differentiation assay is the formation of embryoid bodies (EBs) *via* hanging drop culture in medium without mLIF. On day 0, droplets of 20 µl of a cell suspension (3.75×10^4 cells/ml), with or without test sample, were placed between the well borders on the inner side of the lid of a 96-well plate. The wells of the 96-well plate were filled with 250 µl of PBSto create an optimal humidity and to prevent evaporation of the hanging drops. Sterile caps of Eppendorf tubes were placed in the corner of the plates in order to prevent direct contact of the drops with the plate and the plate was subsequently sealed with Micropore tape (3M, Neuss, Germany) to prevent evaporation of the hanging drops. The hanging drop cultures were incubated for three days at 37 °C and 5% CO_2. On day 3, the resulting EBs were transferred to 60×15 mm bacteriological petri dishes (Greiner Bio-One) containing 5 ml medium, with or without test samples. The petri dishes were incubated for another 2 days at 37 °C and 5% CO_2 in the presence or absence of the test extracts. On day 5, the EBs were transferred to a 24-well plate (Corning) (1 EB/well), containing 1 ml medium with or without test extract. The EBs in 24-well plates were then incubated for 5 days at 37 °C and 5% CO_2. On day 10, the number of wells containing contracting cardiomyocytes was determined by visual inspection using a light microscope. The concentration of solvent in the medium was kept at 0.25%(v/v) DMSO. A solvent control and positive control of 5-fluorouracil (Sigma-Aldrich), final concentration 0.065 µg/ml (0.5 µM) were included in each experiment. This assay was considered valid if the solvent control had at least 21 out of 24 wells that contained contracting cardiomyocytes. Inhibition of differentiation by the test extracts was presented as the fraction of total EBs plated in the 24-well plate that contained beating cardiomyocytes and at least three independent experiments were done for each test extract.

2.9. Zebrafish embryotoxicity (ZET) Test

The ZET assay was performed considering the principles of the OECD 236 guideline [36] and the method described by Beekhuijzen *et al*., (2015) [37]. Zebrafish (*Danio rerio*) wild-type AB line eggs produced *via* group spawning were sorted at about 1-3 hours post fertilization (hpf) in petri dishes using a disposable plastic pipette under the microscope. Since not all the eggs were fertilized, only the fertilized eggs with normal development stages were selected and placed in the incubator until exposure at 4 hpf. The test was done in 24-well plates. Each plate was used for exposure of embryos to one concentration of test extract. In every 24-well plate, 20 wells were filled with 2 ml of exposure medium (one concentration of test sample) while 4 wells were filled with 2 ml egg water for use as internal control. One embryo of the selected fertilized eggs was transferred to every well using a disposable plastic pipette. Methanol extracts of *M. subcordata* fruit, leaf, root, and seed parts were re-dissolved in DMSO and tested at final concentrations of 150, 375, 750, 1125, and 1500 milligrams dry weight per litre (mgDW/L) added from 600 gDW/L stock solution in DMSO, the final DMSO concentration was 0.25% (v/v). Plates were then sealed with self-adhesive film cover to prevent further evaporation of test compound throughout the exposure period (up to 96 hpf). Plates were incubated at 26 ºC with a photo period of 14 hours light:10 hours dark. To ensure the validity of each of three independent experiments, positive, negative, and solvent controls were included in every experiment. 3,4-Dichloroaniline (4.0 mg/L) (Sigma–Aldrich), egg water, and DMSO (0.25%) were used as positive, negative and solvent controls, respectively. The test was done in 5 days starting from the day of exposure (Day 0) and four follow up days (Days 1-4). At the end of the exposure period, acute toxicity (OECD 236, 2013) and general morphology scores (GMS) (Beekhuijzen *et al*., 2015) were recorded every 24hr. The GMS was determined based on the scoring system with 12 developmental endpoints described by Beekhuijzen *et al*., (2015) [37] while acute toxicity (lethality) was determined based on a positive outcome in any of four apical observations: (i) coagulation of fertilized egg, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heartbeat [36]. In this GMS system, there are increasing numbers of indicators for morphology scoring at every observation time point, from 24 hpf to 96 hpf. A lower GMS score indicates a higher degree of inhibition of the zebrafish embryo development in the ZET.

2.10. Data analysis

For each assay, at least three independent experiments were performed. Initial data analysis was done using Microsoft Excel 2016. Data from the ZET and EST assays were expressed as average scores while data from reporter gene assays were expressed as fold changes over the

solvent control and for all assays, results are presented as mean values ± SEM. Statistical significance was assessed using IBM SPSS Statistics 23. Results were compared by one-way analysis of variance (ANOVA) followed by Tukey HSD Post Hoc tests. Results with $p < 0.05$ level of mean difference were considered significant. Graphing was made using GraphPad Prism software version 5.0 (California, USA). For the ZET and EST, nonlinear regression (curve fit) graphs of log(inhibitor) vs normalized response plots of mean and SEM were generated along with result sheets containing IC_{50} values (representing concentrations that decrease a given endpoint response by 50%).

3. Results

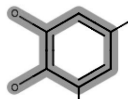
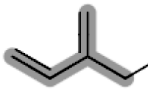
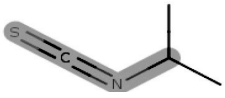
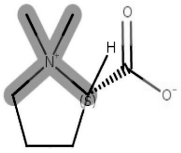
3.1. LC-MS based metabolomics profiling

LC/MS data and MAGMa software based structural annotation revealed glucosinolates, alkaloids and amines as the common secondary metabolites in all the analysed extracts although other phytochemical groups such as phenolic compounds, terpenoids and iridoids were also detected in the leaf extract. Further definite identification confirmed the presence of glucosinolates (glucolepidin and glucobrassicin) and alkaloids or amines (stachydrine and trigonelline) as well as the possible absence of pyrrolizidine alkaloids. Moreover, although standard glucocapparin was not commercially available to confirm definite identification, the LC-MS/MS-MRM data supported its presence in all extracts.

3.2. Derek Nexus toxicity predictions

Derek prediction on the tentatively identified candidates indicated several alerts (endpoints) of toxicity hazards including genotoxicity and developmental toxicity with most of the annotated candidates that reflected toxicity hazards being detected in the leaf extract. **Table S1** (supplementary materials) provides a detailed overview of all candidate constituents that fired structural alerts, **Fig S1** (supplementary materials) shows examples of candidates as detected by LC/MS-MAGMa along with their toxicophores as detected by Derek predicition, while **Table 1** shows some examples of target toxicophores in these toxicity predictions. Candidate molecules for which their definite identification in the extracts could be confirmed, such as glucosinolates and quaternary alkaloids both characterizing the Capparidaceae family [39] to which the study plant belongs, are part of the Derek predictions. For the glucosinolates, a carcinogenicity alert of open (no supporting or opposing evidence) likelihood level was fired. Isothiocyanates which are breakdown products of glucosinolates, raised a plausible alert for chromosome damage *in vitro* in mammalian systems, mutagenicity *in vitro* in bacteria, and

Table 1. Summary of some examples of toxicophores (shaded grey) in candidate constituents of *M. subcordata* methanol extracts

Alerts (Toxicophores)	Description of Derek Nexus toxicity prediction	
	Prediction ⇒Alert fired (Endpoint)	**Likelihood**
 Catechol	Carcinogenicity in mammal	Plausible
	Chromosome damage *in vitro* in mammal	Plausible
 Conjugated alkene*	Carcinogenicity in mammal	Plausible
	Hepatotoxicity in mammal	Plausible
 Epoxide*	Carcinogenicity in mammal	Plausible
	Chromosome damage *in vitro* in mammal	Plausible
	Chromosome damage *in vivo* in mammal	Plausible
	Developmental toxicity in mammal	Plausible
	Irritation (of the eye and skin) in mammal	Plausible
	Mutagenicity in vitro in bacterium	Plausible
	Mutagenicity in vitro in mammal	Plausible
	Mutagenicity in vivo in mammal	Plausible
 Isothiocyanate	Chromosome damage in vitro in mammal	Plausible
	Mutagenicity in vitro in bacterium	Plausible
	Skin sensitisation in mammal	Plausible
 Quaternary ammonium	Irritation (of the eye and the skin) in mammal Skin sensitisation in mammal	Plausible Equivocal

* represent alerst from candidates detected only in the leaf extract

skin sensitisation in mammals. The quaternary amines were linked to a plausible alert of causing irritation (of the eye and the skin) and an equivocal result for causing skin sensitisation in mammals. This study foccussed on results of the Derek toxicity predictions that raised concerns for genotoxicity and developmental toxicity, especially for some candidates in the leaf extract (**Fig S1**), while in the other extracts isothiocyanates formed from glucosinolates may raise a concern for genotoxicity.

3.3. The Ames test

The Ames test indicated negative results (fold revertant colony count <2) (**Fig.1a-f**) for all the extracts at the tested concentrations while the positive controls, 4-nitroquinoline-N-oxide (0.01 µg/plate) and 2-aminoanthracene (2-AA, 1.0 µg/plate) (**Fig.1e&f**) show significant ($p<0.001$) positive mutagenicity. Because exposure to the higher concentrations (30 and 60 mgDW/plate) of the leaf extract with metabolic activation resulted in a fold change in colony counts of less than 1 compared to the solvent control, which may indicate cytotoxicity/ bactericidal effect, the plates were inspected under the microscope for any clearing or diminution of the background lawn [33] and the test for the leaf extract was repeated with lower concentrations (7.5, 15, and 30 mgDW/plate). Thus, results of the leaf extract also include a concentration of 7.5 mgDW/plate and no significant decline in fold reverentant colony count was observed for the 30 gDW/plate concentration in the repeated test (**Fig.1b**). Both the inspection and results of the repeated test showed no meaningful difference in fold change in colony counts apart from random variation indicating that the absence of obvious mutagenicity was not due to cytotoxicity. The Ames test was first done with TA98 strain and then similar tests with TA100 at the highest non-cytotoxic concentration for all the extracts (30 gDW/plate) also resulted in negative results for mutagenicity in this strain (**Fig.1f**).

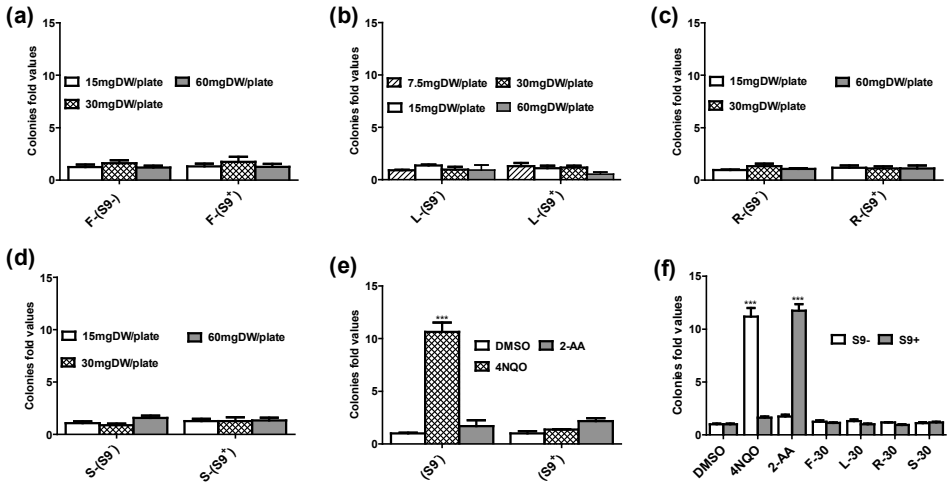


Figure 1. Ames test performed with (S9+) and without (S9-) metabolic activation, with *Salmonella typhimurium* TA98 for (a) fruit(F), (b) leaf(L), (c) root(R), and (d) seed(S) extracts of *M. subcordata*, using (e) DMSO as solvent control, 4-nitroquinoline-N-oxide (4NQO, 0.01 µg/plate) and 2-aminoanthracene (2-AA, 1.0 µg/plate) as positive controls, and (f) showing results of a similar test for the extracts (30 gDW/L) and controls done with *S. typhimurium* TA100. Results are described as fold values of average revertant colonies against the solvent control (DMSO) and are presented as mean ± SEM from three experiments. Asterisks show a significant difference from the solvent control: ***$p < 0.001$.

3.4. DR CALUX assay (AhR CALUX assay)

AhR agonist activities of *M. subcordata* extracts were evaluated by the *in vitro* DR CALUX assay as these endpoints were previously reported to play a useful role in an *in vitro* battery of tests to detect developmental toxicity [31; 39,40]. Results show that upon 6 hr exposure, the fruit, leaf, and seed extracts increased luciferase activity in a concentration dependent manner while the root extract was almost lacking activity in the concentration range tested (**Fig.2a**). A second set of experiments was performed in which luciferase activity from cells exposed for different time periods (6 hr and 24 hr) was quantified in order to check if the AhR induction was transient or persistent. The results from the 6 hr exposure reproduced the previous results while results from the 24 hr exposure showed that all extracts had little or no effect on luciferase activity (**Fig.2b&c**) although the extent of fold induction by TCDD from 24 hr was increased nearly twofold as compared to the 6 hr exposure.

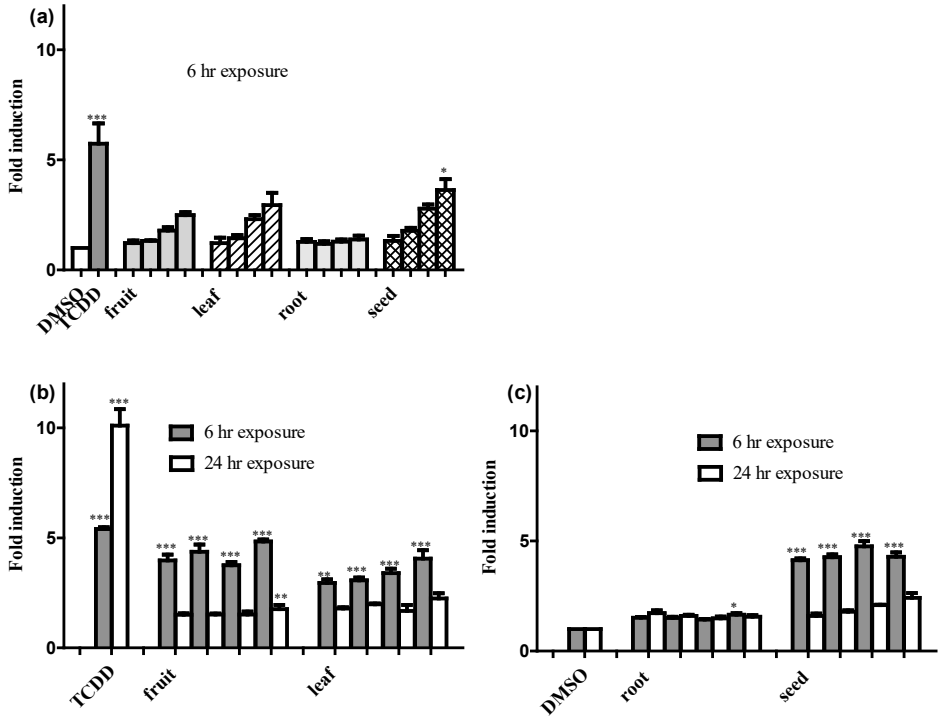


Figure 2. Induction of luciferase activity in rat hepatoma H4IIE-*luc* cells by TCDD at 30 pM (positive control) and different concentrations of fruit, leaf, root, and seed extracts of *M. subcordata* after (a) 6 hr exposure to the extracts at concentrations of 24, 120, 600, and 3000 mgDW/L; (b and c) after 6 hr and 24 hr exposures to the extracts at concentrations of 1.5, 3, 6, and 12 gDW/L. Results are described as fold induction of luciferase activity compared to 0.5% (v/v) DMSO as a solvent control and data are presented as mean ± SEM from at least three independent experiments. Asterisks show a significant difference from the solvent control: *p < 0.05; **p <0.01; ***p < 0.001.

3.5. ERα CALUX and Cytotox CALUX assays

ERα CALUX assay results (**Fig.3**) for *M. subcordata* methanol extracts show that the leaf extract at a concentration of 30 gDW/L increased ERα mediated induction of luciferase activity with an average fold induction of 4.5±0.1 compared to the solvent control. This effect was biologically relevant (fold induction ≥2) [41] but statistically not significant (p>0.05) [42]. In

the same experiment, 17-β-estradiol (E2) at 5 pM displayed an average fold induction of 27.5±2.5 ($p<0.0001$). The fruit, the root, and the seed extracts did not induce luciferase activity in the U2OS ERα assay. **Fig.3** also shows results of parallel screens in the cytotox CALUX assay which reflect luciferase activity similar to the solvent control implying the absence of any cytotoxicity or non-specific changes in luciferase activity as a result of exposure to the tested samples.

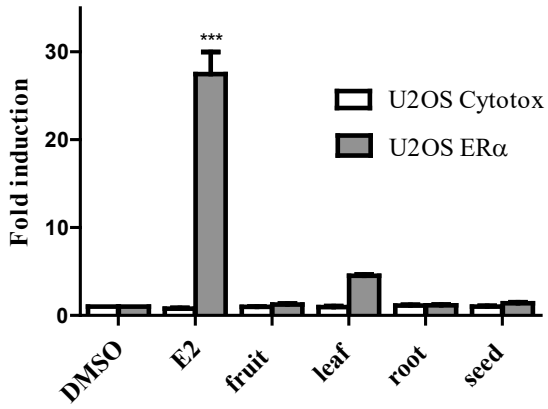


Figure 3. Induction of luciferase activity in U2OS ERα (shaded bars) and U2OS cytotox cells (white bars) cells after 24 hr exposure to 5 pM 17-β-estradiol (E2) (positive control) and *M. subcordata* fruit, leaf, root, and seed extracts at 30 gDW/L. Results are expressed as fold induction of luciferase activity compared to 0.5%(v/v) DMSO as solvent control. Data are presented as mean ± SEM from three independent experiments. Asterisks show a significant difference from the solvent control: ***$p < 0.001$.

3.6. ES-D3 cell viability and cell differentiation assays

Results (**Fig.4a-d**) on the effects of *M. subcordata* extracts on the viability of ES-D3 cells showed that all extracts exhibited relatively similar effects (>89% cell viability) as the solvent control 0.25% (v/v) DMSO, upon both one day and five days exposure, up to the highest tested concentration (1500 mgDW/L). As concentrations showing viability above 80% are generally accepted for further assays [43,44], concentrations up to 1500 mgDW/L of all extracts were considered acceptable in the present study to further characterise effects on ES-D3 cell differentiation. **Fig.4a-d** also show the effects of the extracts on differentiation of ES-D3 cells

into contracting cardiomyocytes, revealing that only the leaf extract significantly ($p<0.001$) inhibited ES-D3 cells differentiation at concentrations ≥750 mgDW/L. 5-fluorouracil (0.5 µM), used as a positive control, showed 85.42±5.51% inhibition of ES-D3 cells differentiation. Conversely, no inhibitory effect was shown by the root extract while the fruit and the seed extracts caused slight effects (26.7% and 16.7% affected fractions, respectively) of inhibition at the highest concentration. The effect was statistically significant ($p<0.05$) for the seed extract but not for the fruit extract although the later seem to show more effect may be because results of repeated experiments of the fruit extract had relatively higher variability compared to those from the seed extract. Only for the leaf extract an IC_{50} value (209 mgDW/L) could be derived from these data, representing the concentration that inhibits ES-D3 cell differentiation into beating cardiomyocytes by 50%. As the extract was non-cytotoxic at the concentrations tested, cytotoxicity may not be expected to contribute to this inhibition of ES-D3 cell differentiation.

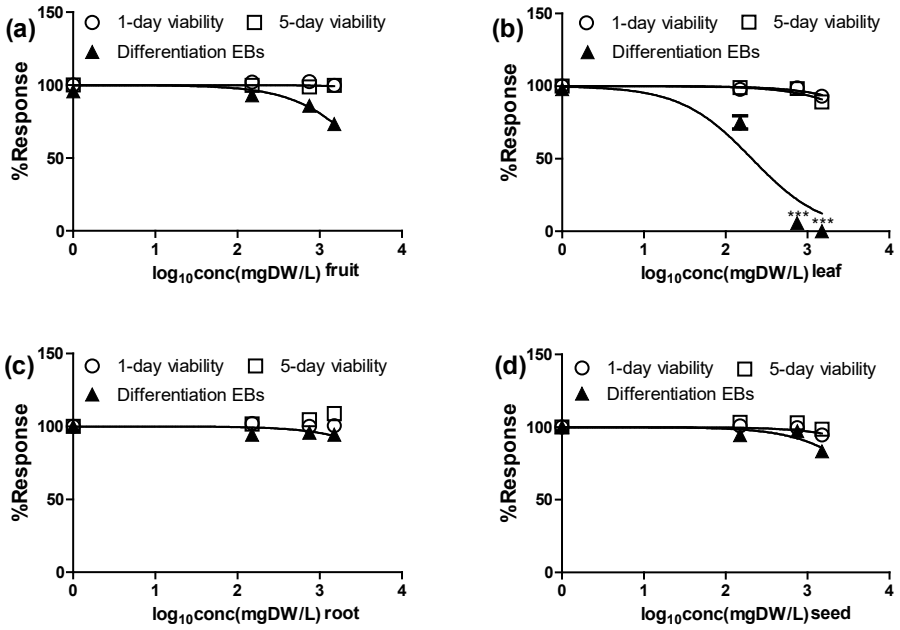


Figure 4. Effects of increasing concentrations (150-1500 mgDW/L) of *M. subcordata* fruit, leaf, root, and seed extracts on ES-D3 cells viability upon 1 day (black circles) and 5 days (black squares) exposures and on inhibition of ES-D3 cells differentiation into contracting cardiomyocytes upon a 10 days exposure (filled black triangles). Results represent mean ±

SEM from at least three independent experiments. Inhibition of ES-D3 cell differentitaion at concentrations where cytotoxicity is not yet observed indicated *in vitro* developmental toxicity. Asterisks show a significant difference from the solvent control: ***p<0.001.

3.7. Zebrafish embryotoxicity test (ZET)

To further assess the *in vitro* acute toxicity and/or developmental toxicity potential of *M. subcordata* extracts, the effects of the extracts on lethality and/or on the general morphology of developing zebrafish embryos were determined by the ZET. Results (**Fig.5a-d**) show that for all concentrations of the tested extracts, the lethality and GMS records were almost overlapping and that in both records, the fruit extract at the highest concentration (1500 mgDW/L, $p<0.05$) and the leaf extract at 750 mgDW/L($p<0.05$) and 1500 mgDW/L($p<0.01$) induced statistically significant effects as compared to the internal control (IC) or negative control (egg water) and the solvent control (0.25%DMSO) whereas the root and the seed extracts did not show statistically significant effects. 3,4-dichloroaniline (4.0 µg/ml), used as a positive control, induced a lethality of 98 ±1.67%. Death of the zebrafish embryos occured either before 24 hpf or immediately after hatching. Besides, occasional cases of malformation of the heart (abdominal and/or pericardial oedema, irregular shape due to edema or aplasia, abnormal heartbeat) [37] in embryos exposed to all test extracts (0 to 5% incidence) but also to 0.25%(v/v)DMSO (3.33% incidence) as well as few curved body/tail in dead embryos exposed to the fruit (1.67% incidence) and the seed (3.33% incidence) extracts were observed. **Fig.6a-g** show examples of normal and abnormal body morphology of some zebrafish embryos exposed to extracts of M. *subcordata*.

Because Selderslaghs *et al*. (2009) [45] reported that DMSO, even at 0.5% (v/v), caused adverse effects on the development of zebrafish embryos and proposed 0.25% (v/v) DMSO (mean% larvae affected <10; not significantly different from controls) as the no observed effect concentration of DMSO, the ≤5% sub-lethal abnormalities (signs of malformation of the heart) (**Fig.6c-d**) observed in the present study were considered not significantly different from the control (0.25% DMSO) and not biologically relevant. Besides, upon exposure to the fruit and seed extracts, some of the embryos that hatched during the time of observation were suffering from stiffness and staggered mobility. Few of the embryos that died immediately after hatching appeared to retain body shape similar to what they were inside the chorion while those that survived maintained normal body shape. Thus, the few cases of curved body/tail (**Fig.6e-g**) in dead embryos exposed to the fruit and seed extracts may be considered as symptoms of acute

toxicity rather than a sign of abnormality since survivors did not show these effects. Also, despite that the fruit, root, and seed extracts had little or no influence on hatchability, the leaf extract showed a significant ($p<0.01$) effect at concentrations ≥750 mgDW/L (**Fig.5b**). An IC_{50} value (249 mgDW/L) could be derived from these data only for the leaf extract, representing the concentration that inhibit hatching by 50% and implying that the IC_{50} values of the other extracts would be beyond the tested concentration range and hence reflecting little or no inhibitory effects on hatching.

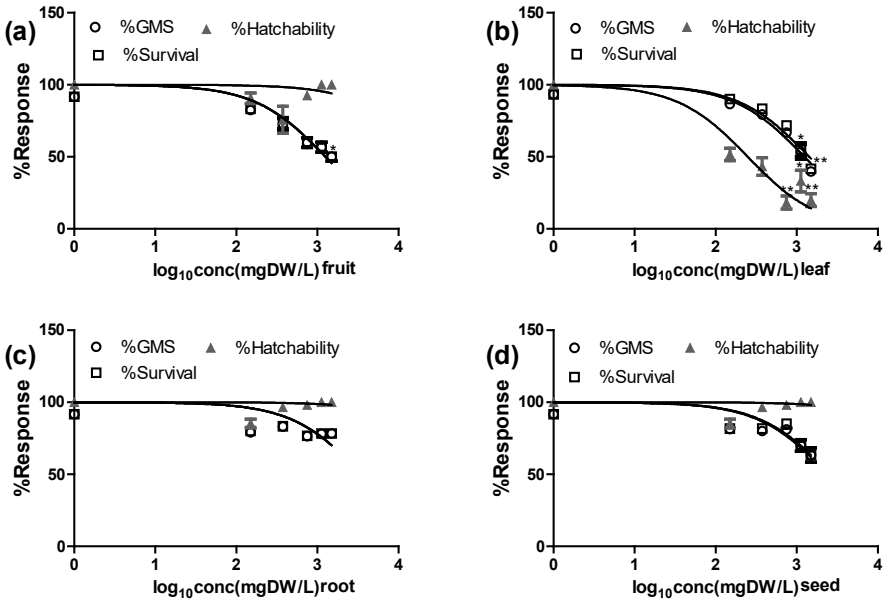


Figure 5. Effects of increasing concentrations (150, 375, 750, 1125, and 1500 mgDW/L) of *M. subcordata* fruit, leaf, root, and seed extracts on zebrafish embryos as compared to solvent control on lethality (described as %survival), general morphology scores (described as %GMS), and %hatchability. Results are described as mean ± SEM of three independent experiments. Asterisks show a significant difference from the solvent control: *$p < 0.05$; **$p <0.01$.

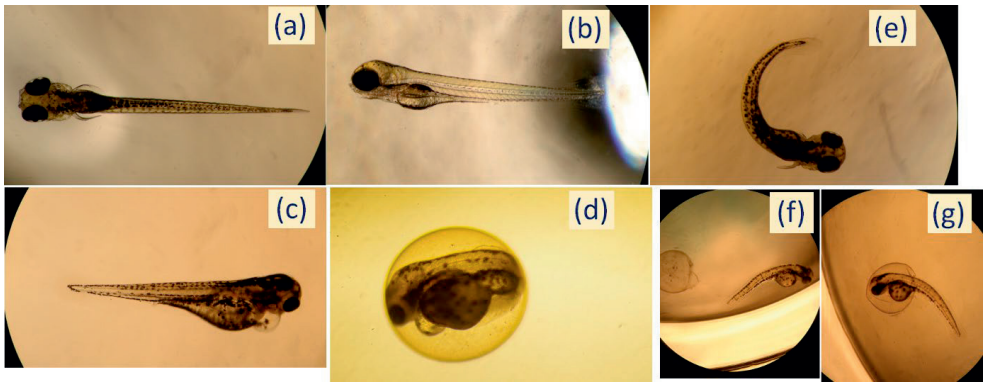


Figure 6. Examples of different morphologies of zebrafish embryos exposed to extracts of *M. subcordata*. (a-b) normal embryos at 96 hpf, (c-d) embryos with signs of malformation of the heart at 96 hpf, and (e-g) curved body/tail in dead embryos between 72-96 hpf.

4. Discussion

The present study applied different *in vitro* tests to assess the genotoxicity and developmental toxicity hazards of methanol extracts of the fruit, leaf, root, and seed parts of *M. subcordata*, a medicinal and (famine) food plant. These endpoints were selected based on the outcomes of a Derek Nexus toxicity prediction, performed on candidate structures obtained from metabolomics profiling of the extracts, and the fact that parts of the plants were reported to be used as abortifacient [20,21].

Although plants are often thought to have anti-mutagenic effects against chemicals and environmental factors, they may also have mutagenic and cytotoxic effects [46]. LC/MS-MAGMa metabolomics profiling plus Derek toxicity predictions showed that candidate constituents for especially the leaf extracts of *M. subcordata* point at genotoxicity hazards. In addition the glucosinolates, detected in all the extracts, may raise a concern because they usually degrade to isothiocyanates (ITCs) and ITCs are suspected of potential genotoxicity in some studies [47] while they were also flagged as potentially genotoxic in the Derek predictions. Results of the present study, however, showed that *M. subcordata* extracts tested negative for mutagenicity in the Ames test, which overrules these concerns. The Ames test has been widely used to assess the mutagenicity of herbal products [46,48]. Although a single test, i.e. the Ames test, cannot cover all genotoxic endpoints and especially does not exclude

concerns in relation to chromosomal damage, *in vitro* bacterial reverse mutation test systems are likely to cover the majority of "critical" endpoints, i.e. DNA-reactivity, of herbal substances [52]. Thus, as the EU guidelines for herbal products define the Ames test as the primary endpoint to judge the genotoxicity [48-50], the results of the present study can be used to support the conclusion that these extracts may not raise a concern with respect to the genotoxicity of *M. subcordata*.

The current study also assessed a second endpoint of concern being developmental toxicity. Herbal remedies, being considered as 'harmless' and 'natural', are often used during pregnancy [51]. Although there are generally insufficient data on the potential embryotoxicity of herbal remedies, some data indicate that their use during the early stages of pregnancy may not be safe for fetal development [51,52]. Therefore, the extracts were tested in a series of *in vitro* assays from a battery of tests developed to assess the *in vitro* developmental toxicity of complex substances [31,39,40]. These tests included the AhR CALUX assay, the ERα CALUX assay, the EST, and the ZET.

AhR, being responsible for the induction of genes that contain a xenobiotic responsive element (XRE, sometimes called a dioxin responsive element) in their promoter regions [53], was implicated in both drug metabolism as well as carcinogenic and toxicological responses against environmental contaminants such as TCDD, but was also shown to be of use in an alternative testing strategy for developmental toxicity [40]. The AhR agonist activities of *M. subcordata* extracts, assessed by the rapid and sensitive *in vitro* CALUX assay [28,30], showed time of exposure dependent variation. The AhR regulates both adaptive and toxic responses [54]. Reports state that transient AhR activation by molecules like 6-formylindolo[3,2-b]carbazole (FICZ), an endogenous ligand with greater binding affinity than TCDD, may be vital for the putative role of the AhR in cell homeostasis [55,56] while sustained AhR activation by molecules like TCDD culminates in toxic responses [54]. Unlike TCDD mediated AhR activation, the AhR activation by *M. subcordata* extracts was of short duration resulting in a transient effect indicating that dioxin like toxicity by the extracts may be limited if not absent. However, AhR activation upregulates transcription of many genes, including those encoding members of the xenobiotic - metabolizing cytochrome P450 1 family of enzymes (CYP1s) [57] and induction or inhibition of CYP enzyme catalysed drug metabolism is among the most common pharmacokinetic interactions responsible for herb drug interactions (HDIs) that result in several significant HDI related adverse health effects [7,58,59]. Therefore, possible adverse

HDIs arising from modulation of the function of the AhR CYP system may be of relevance in cases of concurrent uses of certain drugs and the fruit, leaf, and seed extracts of *M. subcordata*.

Botanical related developmental toxicity may also arise from the endocrine-disrupting (ED) potential of certain plant metabolites, called phytoestrogens [60]. Many ED chemicals adversely impact estrogenic signalling by interacting with estrogen receptors [61]. Although various beneficial health effects have been ascribed to phytoestrogens, their (anti)oestrogenic properties have also raised concerns since they might act as endocrine disruptors, indicating a potential to cause adverse health effects including developmental toxicity [62]. In this regard, only the leaf extract of *M. subcordata* induced some increase in luciferase activity (**Fig.3**) in the ERα CALUX assay, implying possible beneficial/adverse health effects while the activity of fruit, root, and seed extracts was similar to that of the solvent control implying a possible absence of agonistic estrogen activity.

In the EST assay, only the leaf extract of *M. subcordata* indicated possible developmental toxicity since it exhibited significant ($p<0.001$) inhibition of differentiation of ES-D3 cells into contracting cardiomyocytes at non cytotoxic concentrations (**Fig.4b**). In the ZET test, the concentration response curves for lethality and GMS were quite similar (**Fig.5a-d**) suggesting that the GMS scores mostly came from acute toxicity (lethality) effects of the extracts rather than from abnormality endpoints. Malformation of the heart may indicate a teratogenicity endpoint [37] and this effect was observed in a very few (≤5%) embryos upon exposure to all the tested extracts but also upon exposure to the DMSO solvent control, signifying the absence of a significant difference from the control [45]. However, the leaf extract significantly ($p<0.01$) inhibited hatching (**Fig.5b**) which was a major sub-lethal effect by the leaf extract but not induced by the fruit, root, and seed extracts or the solvent control. Hatching is part of the GMS but it is not an endpoint used to measure lethality. It ensures exposure of the embryo without a potential barrier function of the chorion, and as such may help data interpretation [36]. Although David *et al*., (2016) [63] reported that delay or failure to hatch may indicate teratogenic effects, this effect was not part of teratogenic endpoints as described by Beekhuijzen *et al*. (2015) [37] to assess developmental toxicity. Accordingly, the fruit, root, and seed extracts did not show any obvious developmental toxicity [37] apart from acute toxicity [36] but the leaf extract may be suspected of posing such a hazard. Besides, the failure to hatch of the embryos exposed to the leaf extract might have compromised complete scoring

of some endpoints such as deformed body shape which were scored on only the small fraction of hatched embryos.

To sum up, various ethno-medicinal claims on *M. subcordata* may point towards its potential uses as source of herbal medicine or functional or famine food. The different *in vitro* tests of the present study, summarized in **Table 2**, show that all extracts test negative in the Ames test with strain TA98 and TA100, both with and without metabolic activation and hence do not raise a concern for genotoxic hazard [49]; the root extract was apparently devoid of activity in all tests suggesting that further studies may consider the root as potential food item; the fruit and seed extracts seem to have minor toxicity effects except for the fact that they cause strong but transient AhR activation implying caution of, at least, HDI related adverse effects; the leaf extract showed effects in almost all tests except the Ames test indicating its potential multiple biological activities. Hence further studies considering its development and use as potential herbal medicine should also include an adequate safety and risk assessment focussing on at least its potential developmental toxicity. The overall results of the present study do not provide adverse-health effect based counter indications for the ethno-medicinal claim in the plant collection area, northern Ethiopia, that the root tuber part of the plant is a preferred antimalarial remedy for children and pregnant women when there is a fear that other herbal remedies may harm children or pregnant women. This is also in line with the general assumption that a long history of use of a botanical material in traditional medicine is an indicator of lack of obvious toxicity [19].

Table 2. Summary of negative (-) or positive (+) responses to a battery of toxicity tests applied in the present study to assess possible genotoxicity and developmental toxicity hazards of *M. subcordata* extracts.

Assays/ Tests	**Endpoints**	**Response**			
		Fruit	**Leaf**	**Root**	**Seed**
Ames	Mutagenicity	-	-	-	-
ERα	Estrogenic agonist activity	-	+	-	-
AhR	AhR agonist activity	+	+	-	+
EST	Inhibition of embryonic stem cell differentiation	-	+	-	-
ZET	Acute toxicity	+	+	-	-
	Hatching inhibition	-	+	-	-

5. Conclusion

In conclusion, *M. subcordata* leaf extract showed a potential of multiple biological activities including activation of the aryl hydrocarbon receptor, activation of estrogen receptor alpha mediated gene expression, inhibition of embryonic ES-D3 stem cell differentiation into contracting cardiomyocytes, inhibition of hatching of zebrafish embryos, and acute toxicity to zebrafish embryos. These activities may imply potential pharmacological and/or toxicological consequences including developmental toxicity by the leaf extract. The fruit extract showed some cytotoxicity to zebrafish embryos and both the fruit and seed extracts showed induction of the aryl hydrocarbon receptor while the root extract was almost devoid of activity. Safe use of the fruit, root and seed extracts may be expected because hazards of genotoxicity and developmental toxicity as detected in the series of *in vitro* assays applied in the present study appeared limited if not absent. Yet, caution of herbal drug interactions arising from aryl hydrocarbon receptor activation may be suggested in cases of concurrent uses of the fruit, leaf, and seed extracts with certain drugs.

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Conflicts of Interest

The authors declare no conflict of interest.

Appendix: Supporting Information

Supporting information can be found at the end of this chapter

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Appendix: **Fig S1 (Supporting material)**

Figure S1. Showing examples of candidates constituents of *M. subcordata* as detected by LC/MS-MAGMa along with their toxicophores (shaded substructure) as detected by Derek Nexus predicition

Table S1. Summary of Derek Nexus toxicity predictions on tentatively identified candidates of Maerua subcordata methanol extracts. Alerts with likelihood level (certain, probable, and plausible) were considered active otherwise inactive. Candidates that fired alerts with likelihood level of plausible or *probable are presented in this table.

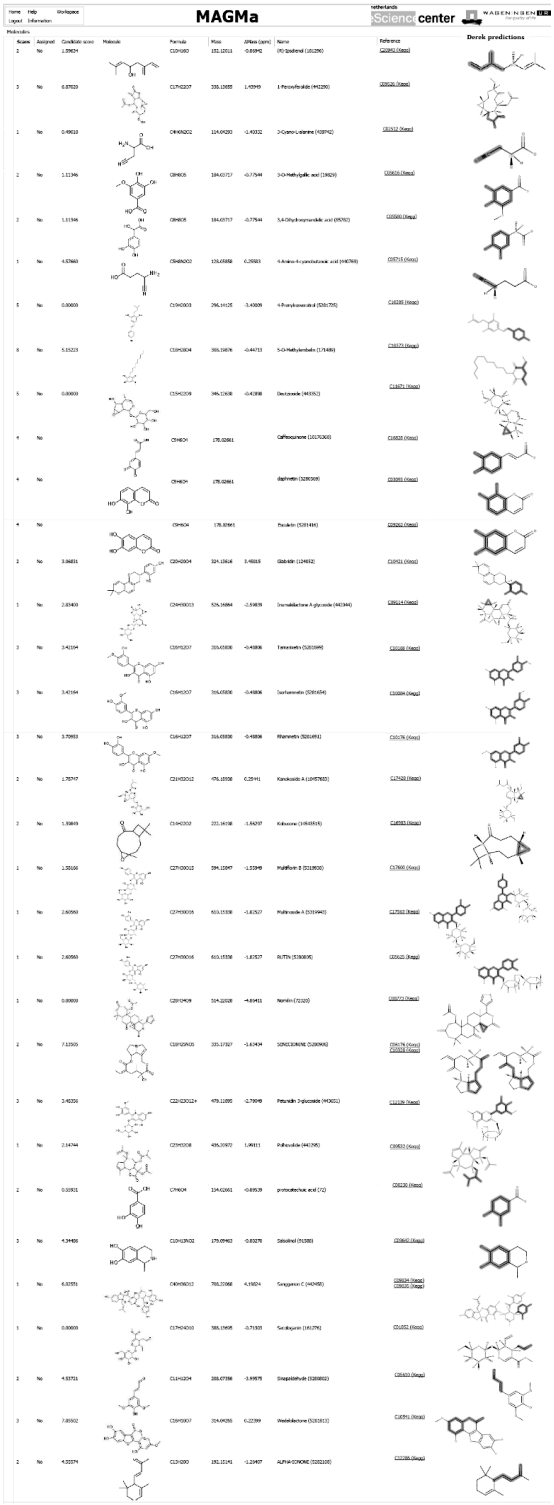

Figure S1

S1 Table. Summary of Derek Nexus toxicity predictions on tentatively identified candidates of *Maerua subcordata* methanol extracts. Alerts with likelihood level (certain, probable, and plausible) were considered active otherwise inactive. Candidates that fired alerts with likelihood level of plausible or *probable are presented in this table.

Alert fired (Endpoint)	Structural alerts matched/toxicophores	Candidate molecules containing the fired structural alert
Carcinogenicity	Catechol	3-O-Methylgallic acid; 3,4-dihydroxypyruvate; 3,4-dihydroxymandelic acid; daphnetin; esculetin; protocatechuic acid; salsolinol
	Conjugated alkene	(R)-Ipsdienol
	α,β-Unsaturated aldehyde, ketone/imine	Caffeoquinone; megaphone
	Epoxide	Deutzioside; inumakilactone A glycoside; kanokoside A
	Extrapolation from thyroid toxicity	Glabridin; sanggenon C
	Pyrroline or pyrroline N-oxide ester, pyrrole ester or pyrrole alcohol, or pyrrolizidine alkaloid	Senecionine
Chromosome damage *in vitro*	Catechol	3-O-Methylgallic acid; 3,4-dihydroxypyruvate; 3,4-dihydroxymandelic acid; 1-(3,4-dihydroxyphenyl)-5-hydroxy-3-decanone; bracteatin 6-O-glucoside; astilbin; daphnetin; esculetin; multinoside A; norathriol; norswetianin; petunidin-3-glucoside; Protocatechuic acid; Quercetin 3-O-rhamnoside 7-O-glucoside; rhamnetin; rutin; salsolinol; veronicastroside; wedelolactone
	Hydroperoxide; α,β-unsaturated ester or thioester	1-Peroxyferolide
	Alkylphenol	4-Hydroxyphenylacetylglycine; γ-glutamyltyramine; L-tyrosine methyl ester; portulacaxanthin II; tyramine
	4-Hydroxystilbene	4-Prenyresveratrol

	Alkyl aldehyde or precursor	10-Hydroxymorroniside; aucubin; deoxyloganin; geniposide; lamiide; secologanin; verbenalin
	α,β-Unsaturated ketone	*α-Ionone
	Epoxide; alkyl aldehyde or precursor	Deutzioside; kanokoside A
	α,β-Unsaturated ester or thioester	Guvacoline; platyphylline; schisantherin B
	Epoxide	Inumakilactone A glycoside
	Pyrroline ester, pyrroline N-oxide ester or pyrrole ester; α,β-unsaturated ester	Senecionine; senecivernine
	Flavonoid	Tamarixetin
Chromosome damage *in vivo*	Epoxide	Deutzioside; inumakilactone A glycoside; kanokoside A
	Flavonoid	Isorhamnetin
Cyanide type effect	Nitrile compounds	3-Cyano-L-alanine; 4-amino-4-cyanobutanoic acid; dhurrin
Developmental toxicity	Epoxide	1-Peroxyferolide; deutzioside; inumakilactone A glycoside; kanokoside A; kobusone; nomilin
Hepatotoxicity	para-Alkylphenol or derivative	3,4-Dihydroxypyruvate; 1-(3,4-dihydroxyphenyl)-5-hydroxy-3-decanone; 4-Hydroxyphenylacetylglycine; Gamma-glutamyltyramine; Portulacaxanthin II
	Conjugated alkene	(R)-Ipsdienol
	Organic peroxide	1-Peroxyferolide
	Aniline or precursor	Damascenine
	Furan	Glycoperine; nomilin
	Phenylethylamine or derivative	L-Tyrosine methyl ester; tyramine
	Quinoline	Quinidinone; quinine
	Pyrroline or pyrrole ester	Senecionine; senecivernine
HERG channel inhibitor *in vitro*	HERG Pharmacophore I	Aspidospermatine; coronaridine; elaeocarpidine; quinidinone; quinine
	HERG Pharmacophore II	Calligonine; granisetron; N,N-dimethyl-5-methoxytryptamine
Irritation (of the Eye)	Quaternary ammonium salt	3-Hydroxystachydrine; betonicine; bufotenidine; γ-butyrobetaine; homostachydrine; stachydrine
	α,β-Unsaturated ester; Epoxide; Alkyl hydroperoxide	1-Peroxyferolide

	alpha,beta-Unsaturated aldehyde	Cinnamaldehyde; Sinapaldehyde
	Epoxide	Deutzioside; inumakilactone A glycoside; kanokoside A; kobusone; nomilin
	α,β-Unsaturated ester	Senecivernine
Irritation (of the Skin)	Quaternary ammonium salt	3-Hydroxystachydrine; betonicine; bufotenidine; γ-butyrobetaine; homostachydrine; stachydrine
	α,β-Unsaturated ester; epoxide; alkyl hydroperoxide	1-Peroxyferolide
	α,β-Unsaturated aldehyde	Cinnamaldehyde; sinapaldehyde
	Epoxide	Deutzioside; inumakilactone A glycoside; kanokoside A; kobusone; nomilin;
	α,β-Unsaturated ester	Senecivernine
Irritation (of the respiratory tract)	Alkyl hydroperoxide	1-Peroxyferolide
	α,β-Unsaturated aldehyde	Cinnamaldehyde; sinapaldehyde
Irritation (gastrointestinal tract)	α-Substituted propionic acid or ester	Polhovolide
Mutagenicity *in* vitro in bacterium	Hydroperoxide	1-Peroxyferolide
	Alkyl aldehyde or precursor	10-Hydroxymorroniside; secologanin
	Quinone	Caffeoquinone
	α,β-Unsaturated aldehyde or precursor	Cinnamaldehyde; sinapaldehyde
	1,3-Dihydroxyxanthone or derivative	Demethylbellidifolin; norathriol; *norswetianin
	Epoxide	Deutzioside; inumakilactone A glycoside; kanokoside A
	Flavonol	Isorhamnetin; multiflorin B; multinoside A; quercetin 3-O-rhamnoside 7-O-glucoside; *rhamnetin; rutin; tamarixetin
Mutagenicity *in vitro* in mammals	Alkyl aldehyde or precursor	10-Hydroxymorroniside; aucubin; deoxyloganin; deutzioside; geniposide; kanokoside A; lamiide; secologanin; verbenalin
Mutagenicity *in* vivo	Epoxide	Inumakilactone A glycoside; kanokoside A
Non-specific genotoxicity *in vitro*	Alkyl aldehyde or precursor	10-Hydroxymorroniside; aucubin; deoxyloganin; deutzioside; geniposide; kanokoside A; lamiide; secologanin; verbenalin
Ocular toxicity	Tropane derivatives	(6S)-Hydroxyhyocyamine; convolamine

Oestrogen receptor modulation	2-Phenyltetralin derivatieve	Glabridin
	Hydroxy-napthalene or derivative	Isorhamnetin; multiflorin B; multinoside A; petunidin-3-glucoside; rutin; tamarixetin
Photoallergenicity	Diaryl ketone	Arborinine; demethylbellidifolin; norathriol; norswetianin
	Coumarin	Daphnetin; esculetin
Skin sensitization	1,2-Dihydroxybenzene or derivatieve	Feruloylagmatine; (+)-syringaresinole; 1-(3,4-dihydroxyphenyl)-5-hydroxy-3-decanone; 1-O-feruloyl-beta-D-glucose; 1-O-sinapoyl beta-D-glucoside; 1-O-vanilloyl-beta-D-glucoside; anhalamine; arborinine; aspidoalbine; astilbin; bracteatin 6-O-glucoside; capsiate; diosmin; quercetin 3-O-rhamnoside 7-O-glucoside; salsolinol; veronicastroside
	Epoxide; Allyl hydroperoxide; alpha, beta-Unsaturated ester or precursor	1-Peroxyferolide
	Resorcinol or precursor	4-Prenyldihydropinosylvin; glabridin
	Substituted phenol or precursor; Resorcinol or precursor	4-Prenyresveratrol; isobavachalcone
	Enol ether; resorcinol or precursor	5,7-Dihydroxychromone
	Quinone	5-O-Methylembelin; caffeoquinone
	Hydroquinone or derivative; Terpenoid	6-geranylgeranyl-2-methylbenzene-1,4-diol
	Hydroquinone or derivative; terpenoid; 1,2-dihydroxybenzene derivative	6-Methoxy-3-methyl-2-all-trans-polyprenyl-1,4-benzoquinol
	α,β-Unsaturated ketone; terpenoid	α-Ionone; gambogic acid
	Alkyl aldehyde precursor; enol ether; α,β-unsaturated ester or precursor	10-Hydroxymorroniside; deoxyloganin ; geniposide; lamiide ; verbenalin
	Alkyl aldehyde precursor; enol ether	Aucubin
	Aromatic primary or secondary amine	Anthranilic acid /4-aminobenzoic acid; damascenine
	α,β-Unsaturated aldehyde or precursor	Cinnamaldehyde
	1,2-Dihydroxybenzene or derivative; dihydroxy coumarin	*Daphnetin; *esculetin
	α,β-Unsaturated ester or precursor	Guvacoline; senecionine; senecivernine
	Epoxide	Inumakilactone A glycoside; *Kobusone; Nomilin

	Conjugated alkene	(R)-Ipsdienol
	Resorcinol or precursor; 1,2-Dihydroxybenzene derivative	Isorhamnetin; multinoside A; norathriol; norswetianin; petunidin-3-glucoside; rhamnetin; rutin; tamarixetin
	Alkyl aldehyde precursor; enol ether; epoxide	Kanokoside A
	α,β-Unsaturated ketone or precursor	Megaphone
	α,β-Unsaturated ester or thioester	Platyphylline; schisantherin B
	Resorcinol or precursor	Sanggenon C
	Aldehyde; alkyl aldehyde precursor; enol ether; α,β-unsaturated ester or precursor	Secologanin
	α,β-Unsaturated aldehyde or precursor; 1,2-dihydroxybenzene derivative	Sinapaldehyde
	Phenyl ester	Wedelolactone
Teratogenicity	Nitrile compounds	3-Cyano-L-alanine, 4-amino-4-cyanobutanoic acid
	Phthalate mono- or di-ester	2-Ethylhexyl phthalate; diisooctyl phthalate/di-n-octyl phthalate
	Pyrroline ester, pyrroline N-oxide ester, pyrrole ester or pyrrole alcohol	Senecionine; senecivernine
Testicular toxicity	Phthalate mono- or di-ester	2-Ethylhexyl phthalate; diisooctyl phthalate/di-n-octyl phthalate
Thyroid toxicity	Resorcinol or 3-aminophenol	Glabridin; sanggenon C

Chapter 6

General discussion and future perspectives

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1. General discussion

Traditional medicines offer promising alternatives to biomedicine, especially with respect to therapies related to aging disorders, chronic and infectious diseases [1]. Ethnopharmacology is at the crossroad of several disciplines, most notably pharmacology and food science, and it has identified numerous botanical preparations and botanical products that are consumed by certain populations for their health benefits [2,3]. The evolutionary perspective of health and nutrition comprises the co-evolution of the food-medicine continuum of wild gathered and cultivated vegetables. Especially wild gathered food plants are often reported in local and popular traditions to have pharmacological activities and are frequently associated with beneficial effects [1]. Consideration of the dietary contexts of local medicines within a wider perspective is key to advance our knowledge of indigenous health care by providing a framework for laboratory investigations that explore the bioactive potential of botanicals [1,4]. In, **chapter 2** of this thesis, ethnomedicinal data on *Maerua subcordata* (Gilg) DeWolf, a (famine) food and medicinal plant, have been presented that helped to design further laboratory based investigations. The root and leaf parts of the plant are used in traditional medicine to treat infections and wounds. Moreover, the root is used to treat diabetes, high blood pressure, pain, allergic disorders, to improve appetite, as laxative and as an abortifacient. The main ethnobotanical applications of the plant discussed in **chapter 2** may be summarised as in **Fig 1**. Most of the diseases for which *M. subcordata* is claimed to be a remedy, may be linked to pathologies of chronic and infectious disorders implying that botanical preparations from this plant may have potential health benefits to manage or prevent such diseases. However, at the same time, safety concerns should not be overlooked since botanicals may pose possible hazards as was reflected in the European Food Safety Authority (EFSA) compendium on botanicals that may raise concern when used as foods [5]. Botanicals and botanical preparations consist of plant-derived products that may include whole plants or specific plant parts (flower, stem, leaves, bark, root, and fruit) or their extracts, used in dietary supplements and herbal medicines [6,7]. Parallel to the globally increasing exposure to botanicals there are increased expectations from consumers, regulators, and producers on quality and safety of these products [7]. Thus, scientific investigation to identify endpoints that may be used to assess possible benefits or hazards following intake of botanical preparations or parts of *M. subcordata* is warranted.

Focus was on use of selected bioassays that detect endpoints of relevance for the potential benefits and possible hazards. Bioassays applied in ethnopharmacology signify the molecular characteristics and complexities of the disease or symptoms for which an indigenous medicine is used in traditional medicine to a variable depth and extent. However, single in vitro bioassays rarely cope with the complexity of human diseases and ignore the concept of poly-pharmacological synergies [1]. Thus, in the present thesis, a battery of different bioassays was used to identify endpoints for multiple parameters that may be applied to evaluate the potential health benefits and possible hazards related to the use of botanical preparations or parts of *M. subcordata* considering its ethno-medicinal uses. Also, since most of the diseases claimed to be managed by this plant may entail pathologies of chronic and infectious disorders, the selection of bioassays considered the current notion that low-grade chronic inflammation plays a significant role in the initiation, propagation, and development of chronic diseases including diabetes, the metabolic syndrome, and infectious complications [8].

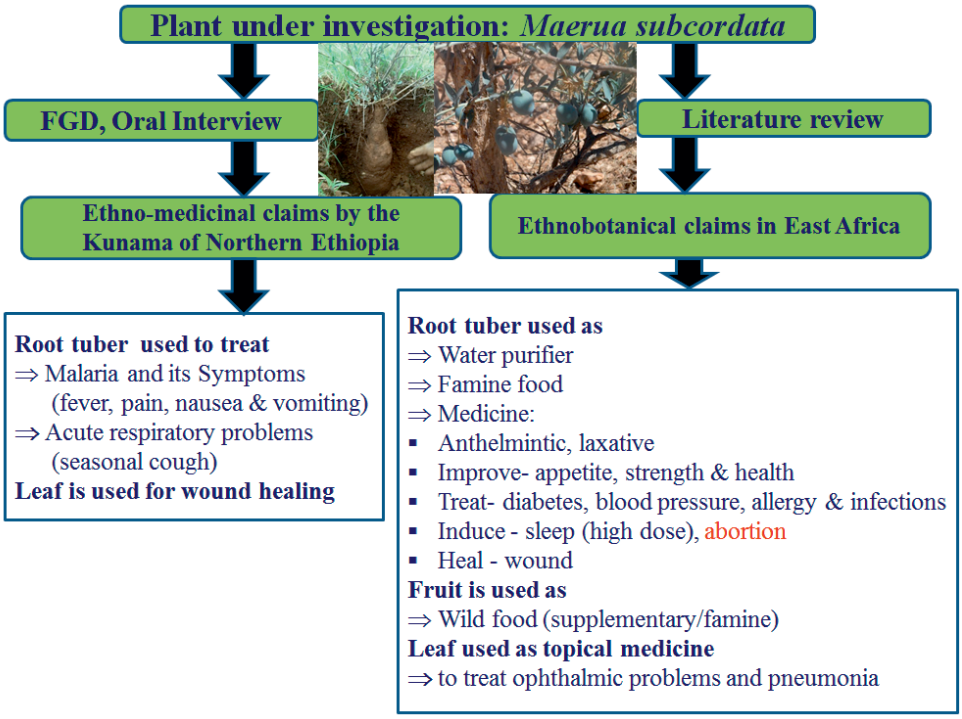

Figure 1: Graphical summary of ethnomedicinal and ethnobotanical uses of *Maerua subcordata*

Besides, overwhelming evidence verified the role of oxidative stress in acute and chronic infection and the associated diseases as well as the impact of major infectious agents on the host redox systems [9]

In addition to considering the use of multi-parameter bioassays, it is also important to consider the multicomponent nature of plant extracts as well as the possible complex interactions of these extracts with biological targets to produce their effect. Plant materials are composed of a wide range of bioactive principles, called phytochemicals that are often responsible for the beneficial or toxic outcomes of the use of plant extracts [10]. Herbal products are usually characterized by a wide therapeutic index as they possess multicomponent mixtures of phytochemicals, which often target many molecular signaling pathways resulting in pleiotropic effects [11,12]. As detailed phytochemical studies on *M. subcordata* are limited, it was decided to perform, in the present study, profiling of candidate constituents in methanol extracts of *M. subcordata* using liquid chromatography coupled to mass spectroscopy (LC-MS) that was followed by 'MS Annotation based on in silico Generated Metabolites' (MAGMa) software structural annotation. This resulted in the identification of some phytochemical groups such as glucosinolates, quaternary ammonium compounds (betaines), and guanidine derivatives. In this way potentially responsible bioactive constituents for some of the endpoints studied in **chapters 3-5** could be identified.

Moreover, whereas the traditional food or medicinal preparations from *M. subcordata* employ either water or butter mixtures, methanol was used to prepare extracts for use in assays and phytochemical analysis as methanol has been generally found to be more efficient in extraction of lower molecular weight compounds [13]. Characteristically, plant extracts are composed of complex mixtures of individual phytochemical constituents and potential contaminants [7,10]. Thus, testing these complex mixtures in in vitro systems pose particularly unique challenges, such as determining appropriate testing concentrations, solubility, and inherent antibacterial or cytotoxicity properties of constituents as well as comparing of results with literature data [7]. Especially, the absence of phytochemical studies of a botanical, which was the case for *M. subcordata*, can make this challenge to be more complex. Thus, metabolomics profiling of the test extracts was necessary. LC-MS can generate comprehensive spectral information of metabolites in crude extracts. To support structural characterization of the many metabolites present in such complex samples, a novel method (http://www.emetabolomics.org/magma) was presented by Ridder and co-workers to automatically process and annotate LC-MS data sets on the basis of candidate molecules from chemical databases; and alternative candidate molecules

are ranked on the basis of calculated matching score and displayed on the user interface [14]. However, this provides only an overall overview on the general phytochemical profile of samples and definite identification of individual compounds of interest requires additional analytical procedures such as use of commercially available standard compounds and co-analysing of samples using LC-MS in multiple reaction monitoring mode, which is cost and time intensive. Therefore, definite identification was made on only a limited number of individual compounds of *M. subcordata* extracts, considering at least one representative of the phytochemical groups identified: glucosinolates, quaternary ammonium compounds, and guanidine derivatives.

The selected bioassays reflected cellular response pathways mediated by transcription factors such as the aryl hydrocarbon receptor (**AhR**), the nuclear factor (erythroid-derived 2)-like 2 (**Nrf2**), the nuclear factor kappaB (**NF-κB**), the peroxisome proliferator-activated receptor gamma (**PPARγ**), and the estrogen receptor alpha (**ERα**). Moreover, the embryonic stem cell test (**EST**) and the zebrafish embryotoxicity test (**ZET**) were included in the test battery to investigate alerts for developmental toxicity as well as the **Ames** test and *in silico* predictions for initial genotoxicity screening. These bioassays were applied and described in **chapters 3-5** and the focus of discussion here is on the holistic implications of these endpoints as related to potential health benefits and/or hazards. The main endpoints from the different assays/tests are summarized in **Table 1** to facilitate straightforward discussions. While the main health related cellular response pathways considered in the present thesis were described in **chapter 1**, **Fig 2** demonstrates a common scientific view of infections, oxidative stress, and inflammation related disease pathologies. It aims to show a simplified and contextualized description of pathologies related to disturbed redox homeostasis so that the different endpoints from different bioassays in the present thesis may be linked to such pathologies and discussed in a holistic context elaborating their implications mainly in maintaining redox homeostasis. This takes into account the assumption that especially in the area where plant collection was made, the ethno-medicinal claims (use against malaria and its symptoms and respiratory problems) may point to a possible role of preparations from *M. subcordata* to relief infection derived inflammatory symptoms (such as pain and fever) and associated oxidative stress.

The use of botanicals for maintaining good health and preventing diseases is well established. Although evidences exist on the beneficial effects of botanicals, their use can only be realised after the verification of their safety and efficacy [15]. Some criteria of EFSA's opinions on the

scientific substantiation of a health claim have implications for the research required to substantiate health claims. However, the complexity of the mechanisms and actions of phytochemicals is not recognised by these criteria used to evaluate proposed health claims, nor by the methodologies used to assess their effects [16]. The well-known disease preventive effect related to dietary intake of foods rich in fruits, vegetables, and unrefined grains has been linked to their phytochemical content that often produces a broad range of physiological effects by interfering with potentially pathologic stress pathways [17,18]. Four general types of cellular stress responses that are linked to the pathogenesis of most major chronic diseases are (1) oxidative, (2) inflammatory, (3) metabolic, and (4) proteotoxic stresses [17]. The first two play a pivotal role [19,20] and were the major focus in this thesis. Several phytochemicals have been approved as health promoters including the breakdown products of glucosinolates (isothyocyanates and indoles), which are linked to the health benefits from consumption of brassica vegetables [21] through suggested multiple mechanisms, including mainly antioxidant and anti-inflammatory effects [16,21].

Table. Summary of effects of *M. subcordata* (fruit, leaf, root, and seed) extracts in different bioassays showing positive (+) or negative (-) responses as presented in chapters (CHs) 3-5.

Assays/ tests	Endpoints	Responses				CH
		Fruit	Leaf	Root	Seed	
AhR	AhR agonist activity	+	+	-	+	5
EpRE	Nrf2 activity (induction)	+	+	-	+	3
PPARγ	PPARγ activity (induction)	+	-	+	+	4
RAW264.7	NF-κB mediated NO production (inhibition)	+	+	+	+	4
ERα	Estrogenic agonist activity	-	+	-	-	5
Ames	Mutagenicity	-	-	-	-	5
EST	Embryonic stem cell differentiation (inhibition)	-	+	-	-	5
ZET	Acute toxicity	+	+	-	-	5
	Hatching inhibition	-	+	-	-	5

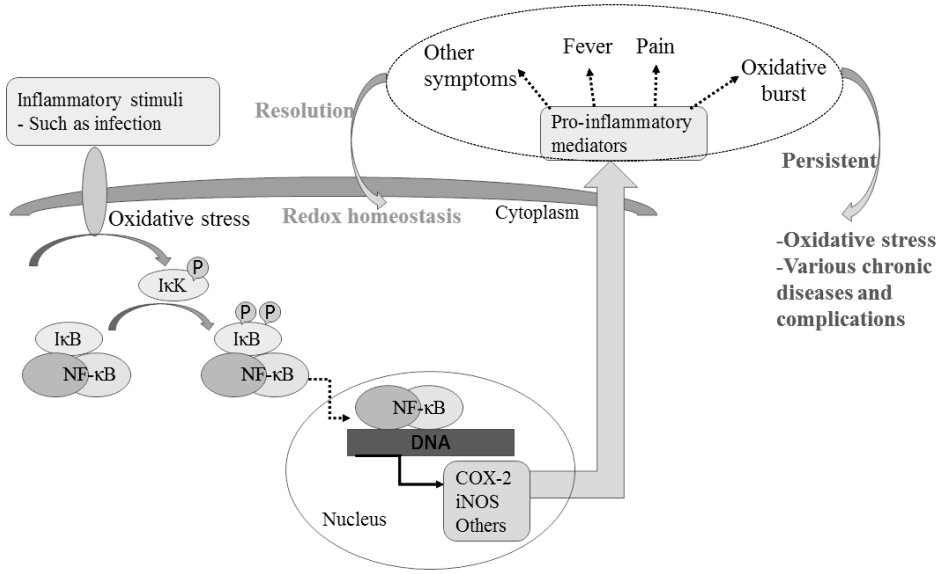

Figure 2. Activation of certain receptors (e.g. toll-like receptor 4) induces a signalling pathway leading to the activation of NF-κB: this involves phosphorylation of IκB kinase (IkK), then phosphorylation of IκB followed by its dissociation from the inactive cytosolic NF-κB. The active NF-κB translocates to the nucleus and initiates transcription of genes for inflammatory mediators such as the inducible cyclooxygenase 2 (COX-2) and inducible nitric oxide synthesis (iNOS). An early inflammation is initiated to resolve an insult from noxious stimulus, but persistent injury results in chronic inflammation and associated diseases.

Phytochemicals help the holistic maintenance of the body's redox homeostasis (the endogenous capacity of cells to continuously deal with challenges that generate electrophiles/oxidants) *via* oxidative feedback mechanisms operating at different level of complexity [20]. Oxidative stress is mainly a biochemical dysregulation of intracellular redox status, whereas inflammation can be the biological response to oxidative stress intended to restore homeostasis [22]. Inflammation is initiated to resolve an insult from noxious stimulus, but persistent injury results in chronic inflammation [23]. It is now widely appreciated that low-grade chronic inflammation plays a significant role in the initiation, propagation, and development of metabolic diseases, mainly in relation to obesity and type 2 diabetes, the metabolic syndrome, cancer, and cardiovascular diseases [8,22,24]. Plant extracts and their phytochemicals can differentially or co-ordinately interfere, especially with pathologic oxidative and inflammatory stress pathways

to produce their effect. This may also apply to candidate constituents of *M. subcordata* extracts taking into account findings of **chapters 3-5**, **table 1,** and discussions below.

In **chapters 3** and **4**, *M. subcordata* extracts and candidate constituents like isothiocyanates were shown to induce Nrf2 activation and inhibition of NF-κB mediated nitric oxide production. Besides, some betaines like stachydrine and guanidine derivatives like agmatine exhibited possible NF-κB inhibition as measured by inhibition of lipopolysaccharide (LPS) induced and iNOS mediated nitric oxide overproduction. Nrf2 and NF-κB are key transcription factors that regulate cellular responses to oxidative stress and inflammation, respectively [25,26]. NF-κB regulates cellular immune responses to infection and higher order oxidative stress by coordinating a pro-inflammatory response [27] whereas Nrf2 may play an important role in defense against oxidative stress by activation of the cellular antioxidant machinery and suppression of pro-inflammatory signalling pathways [28]. Several evidences support the crosstalk between Nrf2 and NF-κB pathways [28]. These two factors are inversely regulated, with activity of one most often accompanied by diminished activity of the other [26]. Also, absence of one may be convoyed by exacerbated activity of the other. For example, the absence of Nrf2 can exacerbate NF-κB activity leading to increased cytokine production, whereas NF-κB can modulate Nrf2 transcription and activity, having both positive and negative effects on the target gene expression [25]. It was verified that Nrf2-knockout mice are more sensitive to inflammatory diseases [29] and carcinogenesis [30], which supports the crucial role of Nrf2 in inflammation and the regulation of pro-inflammatory genes [31]. This pathway plays a major role in health resilience and can be made more robust and responsive by certain dietary factors such as phytochemicals [22]. It was shown that LPS induced NF-κB activation could be attenuated by diverse Nrf2 activators, such as phenethyl isothiocyanate, sulforaphane, and curcumin [28,32]. In this context, *M. subcordata* may provide possible health benefits considering that it contains glucosinolates and the findings of **chapters 3** and **4.** Glucosinolate breakdown products, chiefly indoles and isothiocyanayes, are known for their ability to exert multiple biological activities with multiple potential health benefits. Mainly, the abilities of these phytochemicals to induce cytoprotective genes, mediated by the Nrf2 and AhR transcription factors, and their abilities to repress NF-κB activity are important [33].

As was described in **chapters 3-5**, especially the fruit, leaf and seed extracts of *M. subcordata* demonstrated activation of the **AhR** and **Nrf2** mediated pathways but inhibition of LPS induced nitric oxide production, which is regulated by the **NF-κB** signaling pathway. Besides, the fruit,

root and seed extracts showed activation of the **PPARγ**. This indicates that these plant parts may possess phytochemicals with possible multi-target effects that can be associated to disease preventive and/or therapeutic potential. The potential health benefit of these endpoints can be justified considering the holistic view that phytochemicals can differentially or co-ordinately regulate Nrf2 mediated antioxidant or NF-κB mediated anti-inflammatory signaling pathways as the first line of defense or otherwise induce activator protein-1 (AP-1) mediated apoptosis once the cells have been damaged (**Fig 3A**) [20,22,28,34]. AhR activation can activate or synergize the cytoprotective role of Nrf2 [33,35] while induction of PPARγ can inhibit the NF-κB pathway to produce an anti-inflammatory role [36]. Phytochemicals modulate these pathways and may help cellular adaptive homeostasis to be strong and robust and thereby may play disease preventive roles by boosting the cellular resistance to oxidative stress (**Fig 3B**). Although crosstalk among all the pathways employed in this thesis are reported, the discussions here emphasise on Nrf2 and NF-κB, which are the two crucial response pathways implicated in the pathogenesis of most major chronic diseases including the ones claimed to be treated by the plant under study.

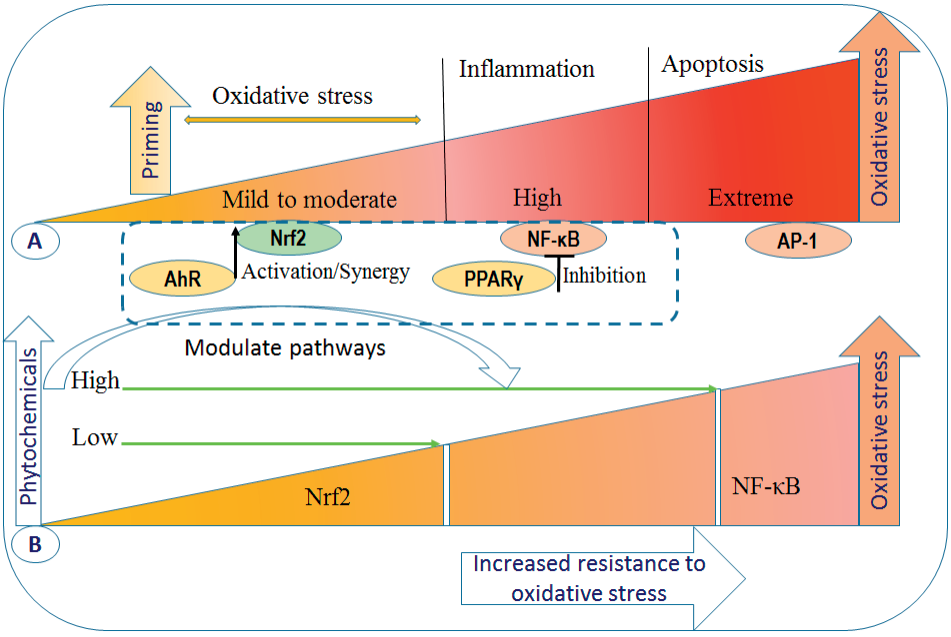

Figure 3. Showing (**A**) differential responses to rising oxidative stress and (**B**) increased cellular resistance to oxidative stress and protection against inflammation by phytochemicals [22].

While the molecular mechanisms involved in the crosstalk between Nrf2 and NF-κB pathways still need to be elucidated [28], some suggested mechanisms have been explained [22,27,31]. In the Keapl/Nrf2/ARE signaling pathway, oxidative triggers release Nrf2 from its negative regulator Keap1 which allows Nrf2 to translocate into the nucleus where it binds to target genes, the antioxidant/electrophile-responsive elements (ARE/EpRE) and induces the expression of antioxidant and detoxification enzymes [22,37]. Equally, the release of NF-κB from its inhibitor protein (IκB) requires the phosphorylation of IκB by cytosolic protein, IκB kinase (IKK) [27]. Freed NF-κB translocates to the nucleus and initiates transcription of genes for inflammatory mediators. Nrf2 and NF-κB influence each other in at least three ways: first, IKK can bind Keap1 and be targeted for ubiquitination. When oxidative triggers release Nrf2 from Keap1, there is an increase in the intracellular pool of unbound Keap1 available to capture more intracellular IKK, thus inhibiting the expression of NF-κB target genes. Hence, this may be one mechanism by which Nrf2 activation is known to inhibit NF-κB activation [22,37]. Second, the late phase inflammatory process induces inflammatory mediators such as COX-2 that catalyses the formation of 15-deoxy-delta-12,14-prostaglandin J2 (15d-PGJ2), a strong electrophile that reacts with Keap1 and activates Nrf2, thus initiating cytoprotective gene transcription with simultaneous inhibition of NF-kB activity [31,38]. Third, both NF-κB and Nrf2 compete for the same transcriptional co-activator, the cyclic-AMP responsive element binding protein (CREB) binding protein (CBP), which promotes DNA binding of both transcription factors [26,31,39]. When NF-κB binds with CBP in a competitive manner, it inhibits the binding of CBP with Nrf2, which leads to the inhibition of Nrf2 transactivation [28,31,40]. Moreover, beyond a certain threshold in the intracellular oxidation status, Nrf2 may promote generation of reactive oxygen species [37] that ultimately result in irreversible oxidation of thiols in Keap1 [41,42]. This would be expected to abrogate the Keap1 inhibition of IKK allowing for an increase in NF-κB activation. NF-κB has been known to inhibit Nrf2, and so this may be the transition point where oxidative stress becomes inflammation [22]. Phytochemicals may help to push this transition point to ensure more robust redox homeostasis. Indeed, isothiocyanates were shown to inhibit IKK/IκB phosphorylation and NF-κB nuclear translocation, thus alleviating NF-κB signaling [28,32].

Moreover, AhR activation and induction of PPARγ by natural products such as *M. subcordata* extracts may play an essential health promoting role by augmenting the function of Nrf2. AhR

activation can activate or synergize Nrf2 activity [33,35] while induction of PPARγ can inhibit the NF-κB pathway [36] and may indirectly help Nrf2 in its cytoprotective role. Also, it was shown that the PPAR*γ* ligand, 15d-PGJ_2, which is a strong Nrf2 activator [38], negatively regulates the cells of the innate and adaptative immune system and presents excellent results in different models of inflammatory diseases [43]. This may imply possible cooperation of PPARγ and Nrf2 in resolving situations of late phase inflammation.

The PPARγ regulates metabolism, adipogenesis, and inflammation pathways [44,45]. PPARγ activation is linked to beneficial health effects, including insulin-sensitization and it acts as a key immunomodulator with anti-inflammatory properties [46,47]. The PPARγ agonists' ability to inhibit inflammatory responses by repressing NF-κB target genes has been linked to the prevention and treatment of the metabolic syndrome and diabetes [48,49]. Thus, induction of PPARγ gene expression or inhibiting NF-κB pathways may likely exert a protective effect against inflammatory chronic diseases [50]. As detailed in **chapter 4**, *M. subcordata* extracts may share such potential health benefits, which supports the use of the plant in traditional medicine to manage infectious and chronic diseases associated to the metabolic syndrome including diabetes.

The AhR mediates the biologic and toxic effects of its ligands [51]. The physiological functions of the AhR may require tightly controlled and transient signaling, whereas sustained AhR signaling may trigger pathological responses [35]. The evolutionary conservation of AhR and its widespread expression in the immune system point to its important physiological functions, which has led to a shift of emphasis from its role in the xenobiotic pathway toward its mode of action in response to physiological ligands. Especially the indole derivatives from tryptophan and the *Brassica* glucosinolate, glucobrassicin are important physiological ligands. Although the underlying mechanism still need to be elucidated, interfering with controlled AhR signaling may trigger many of the adverse effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on immune responses [35]. The AhR signaling is regulated at three levels: proteasomal degradation of the AhR, ligand metabolism by cytochrome P450 (chiefly CYP1A1), and AhR/ARNT (AhR nuclear translocator) complex disruption by AhRR (AhR repressor) [35]. Autoregulatory feedback by the drug-metabolizing CYP enzymes is central to the regulation of AhR signaling. In contrast to TCDD, endogenous ligands such as 6-formylindolo[3,2-b]carbazole (FICZ) or dietary phytochemicals are excellent substrates for the CYP enzymes downstream of AhR [35, 52]. Thus, ligands that are efficiently metabolized by CYPs have been linked to transient AhR activation and subsequent physiological responses. Therefore, although this endpoint was

intended to identify possible hazards of dioxin like effects by *M. subcordata* extracts, the observed transient AhR activation by these extracts may not show such a hazard. However, a possible herbal drug interaction may be cautioned in case of concurrent use, especially of the fruit, leaf, and seed parts.

It was established that there is a fine-tuned crosstalk between AhR and Nrf2, which mutually increase or decrease their activation states [53]. The AhR and Nrf2 are amid the widely studied transcription factors that mediate xenobiotic adaptation [54] by modulating the expression of target genes [55], the xenobiotic-responsive elements (XREs) and the ARE/EpRE, respectively. AhR activation induces different cellular processes including biotransformation of xenobiotics by phase I and II xenobiotic metabolizing enzymes while Nrf2 activation induces antioxidant enzymes and phase II detoxification enzymes [17]. The common beneficial aspects of these two transcription factors is cytoprotection against injurious chemicals. The cooperation of Nrf2 and AhR is multiple. First, reactive oxygen species produced by AhR-mediated phase I biotransformation can stimulate Nrf2. Second, several target genes exhibit ARE and XRE in their promoter and so, their expression can be increased by Nrf2 and AhR, such as NADPH/quinone oxidoreductase 1 (NQO1). Finally, AhR and Nrf2 mutually stimulate their expression by a direct transcriptional activity. Upon xenobiotic exposure, Nrf2 and AhR mediate cytoprotection by inducing the effective enzymes for detoxification and modulating metabolic pathways in order to supply energy to support protective activity. These processes are supposed to be transient and allow the survival of the attacked cells. When the exposure is persistent or repeated, or when the activation of Nrf2 or AhR is chronic, metabolic dysfunction can occur, as it is the case in cancer [56]. Thus, the transient AhR activation by *M. subcordata* extracts may be considered beneficial sharing the cytoprotective role of Nrf2. Glucosinolates are among the constituents that characterize *M. subcordata* [57] and their hydrolysis products, the isothiocyanates were shown to demonstrate chemo preventive action *via* inhibition of phase I and induction of phase II detoxification enzymes by modulating the AhR/Nrf2 signalling pathway [58-60].

In **chapter 5**, the focus was to identify endpoints for the assessment of potential hazards of botanical preparations from *M. subcordata*. Although use of plant products as food supplements or herbal medicines continue to increase worldwide, there still remains a huge research gap on their efficacy, quality and safety aspects [7,61]. With regard to safety, there remain significant data gaps or conflicting data for critical toxicity end points, mainly attributed to an over-reliance on limited historical information rather than empirical testing of these complex botanical

mixtures [7]. A framework that could be applied to assess the safety of a botanical or a botanical preparation has been outlined by EFSA, the application of which depends on certain factors including the nature of the botanical or botanical preparation, its intended uses and levels of use in food supplements, and on whether a long history of food use is evident. When there is lack of a history of food use or other relevant safety data, an assessment of safety generally relies on experimental toxicity data largely derived from investigations in laboratory animals [62]. However, considering the challenges of testing the complex mixtures of phytochemical constituents of botanicals as well as the cost, time, and animal usage constraints, an emphasis has been put in recent year, against in vitro testing approaches to address critical end points where safety data are lacking [7]. Therefore, a battery of in vitro data were planned in **chapter 5**, to identify selected endpoints that may help the hazard assessment of the study plant, which lacks safety data.

In the compendium by EFSA of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements, glucosinolates are mentioned as chemicals of concerns related to the use of *Brassica nigra* (L.) W.D.J.Koch [5]. It was noted that alkyl and indolyl glucosinolates are characteristic constituents of many species of the Capparidaceae family including *Maerua* species. Mainly, glucocapparin is widely distributed throughout many species of this family, including *M. subcordata*, often as the most abundant compound [57]. Glucocaparin hydrolyses to produce methyl isothiocyanate (MITC), which may be suspected as possibly causing genotoxicity and developmental toxicity although based on limited studies. A genotoxic study revealed that MITC induces only marginal effects at extremely high (almost lethal) doses in inner organs in vivo, but it causes DNA-damage at low concentrations in vitro. MITC caused only marginal mutation induction in reversion assays (Ames test) with *Salmonella typhimurium* strains TA100 and TA98. In differential DNA-repair assays with different *Escherichia coli* strains, a pronounced dose-response effect (induction of DNA-damage) was reported at low concentrations (≥4 µg/ml) although addition of a metabolic activation mix (rat liver S9) led to a reduction of these genotoxic effects. Moreover, further in vitro experiments showed that MITC is inactivated by body fluids (saliva, gastric juice) and that its DNA-damaging properties are attenuated by non-enzymatic protein binding. In a micronucleus assay with human hepatoma cells (HepG2), clear cut DNA-damaging effects were observed at exposure concentrations of <5 µg/ml, an observation that was suggested to involve lipid peroxidation processes as exposure of HepG2 cells to MITC led to formation of thiobarbituric acid reactive substances [63]. In addition, two zebrafish tests for developmental

toxicity indicated that MITC tested positive for causing in vitro developmental toxicity [64,65]. The most notable malformation among surviving embryos was a severely twisted notochord, which was not immediately lethal and the embryos continued to grow despite delays in hatching, apparent paralysis, and an inability to feed [64]. These reports, though limited, may indicate that consumption of preparations or parts of *M. subcordata* may pose risks of genotoxicity and developmental toxicity and hence were made the focus of **chapter 5** of this thesis.

In line with these data on MITC in our initial studies, Derek software based genotoxicity predictions on candidate constituents of *M. subcordata* indicated a positive alert for mutagenicity by some constituents, mainly in the leaf extract, including isothiocyanates (ITCs). A Derek toxicity prediction indicated the absence of toxicity alert for glucosinolates, however their likelihood level for carcinogenicity was displayed as open (no supporting or opposing evidence). Yet, the ITCs showed an alert of plausible likelihood level to cause mutagenicity in vitro in bacterium and chromosome damage in vitro in mammal. According to EFSA, in vitro tests covering effects both at gene and chromosome levels are required for the genotoxicity testing of botanicals and botanical preparations, which may include the Ames test and an in vitro test for the detection of chromosomal aberrations [62]. However, bacterial reversion assays with *Salmonella typhimurium* strains TA98 and TA100 tested negative for all tested extracts of *M. subcordata*, which may indicate the absence of potential hazard of mutagenicity by this plant. The EU guidelines for herbal products suggests no further test in case of a negative Ames test, which is the primary endpoint to judge genotoxicity [66]. It may be questioned whether the Ames test is able to detect the activity of low levels of a compound of concern in complex mixtures. A recent analysis revealed that the Ames test can be considered as valuable test for its capacity to detect low levels (0.01 mg kg^{-1}) of highly potent genotoxic carcinogens, although it cannot be used as standalone method to evaluate genotoxicity potential of low level mixture constituents of low potency [67]. Moreover, in vivo tests do not support the suspicion of ITCs as potential genotoxic agents. For example, allyl isothiocyanate was not genotoxic *in vivo* in mice and rats [68] and the overall evaluation of the International Agency for Research on Cancer (IARC) show that allyl isothiocyanate is not classifiable as carcinogenic to humans (Group 3) [69]. Instead, the weight of evidence suggests that ITCs are promising anti-cancer agents and even few ITCs such as sulforaphane and phenethylisothiocyanate have advanced to clinical phase studies [70]. Therefore, the overall opinion may be that the current scientific data do not point to a genotoxicity hazard for *M. subcordata*.

Regarding developmental toxicity, the leaf extract tested positive for inhibition of embryonic stem cell differentiation in the EST test and inhibition of the hatching of zebrafish embryos in the ZET test indicating a possible hazard for developmental toxicity whereas the other extracts (fruit, root, and seed) tested negative. Similar to the case of genotoxicity, existing in vivo studies do not support the suspicion that ITCs may cause developmental toxicity although based on limited data: allyl isothiocyanate did not cause any developmental toxicity in rats, hamsters and rabbits [68]. Interestingly, there is no reported traditional claim on the use of *M. subcordata* leaf part that allow internal administration other than topical applications. Also, the leaf extract was positive towards activation of ERα and AhR pathways. The fruit and seed also showed strong induction of the AhR pathway. Yet, as discussed above, considering the physiological roles of transient AhR activation, which was the case for the extracts of *M. subcordata*, this endpoint was not considered as a hazard in this work. Likewise, a definite conclusion on possible health benefit or risk of ERα activation requires consideration of several factors as ERα is essential for numerous key physiological functions although many adverse effects of estrogens have been indicated to involve the activation of ERα signaling [71,72]. Various beneficial health effects have been ascribed to ERα ligands such as phytoestrogens, but their (anti)estrogenic properties have also raised concerns since they might act as endocrine disruptors, indicating a potential to cause adverse health effects. Thus, a definite conclusion on possible health benefit or risk of phytoestrogens cannot be made [73]. Contrary to the claimed use in Somalia of the root of *M. subcordata* as abortifacient [74,75], our findings indicate no potential hazard for developmental toxicity for this plant part, which is in line with the claim by the Kunama ethnic of northern Ethiopia, where collection of plant material in the present study was made, that the root tuber is used as a safe antimalarial remedy, preferred for children and pregnant women.

2. Concluding summary and future perspectives

The present thesis provided in vitro data that can be used as input background data to plan further investigations tailored to apply ***'Ashkulebya'*** (*M. subcordata*) as a viable local source of potential functional food, which may subsidise food security and/or promote heath, taking both possible benefits and risks into account. Moreover, findings of this study may offer a proof of concept on how to evaluate possible dietary use of wild edible plants by providing scientific data that help to consider potential hazards and benefits, thereby supporting the rational use of botanicals. Taken together, the findings of the present thesis provide in vitro data to guide

further in vitro and/or in vivo studies to realize rational use of preparations or part of *M. subcordata* as summarized graphically in **Figs 4-6**. To this end, the root tuber was found to display weak/no activity in amost all assays/tests indicating that further study may consider it as food item, including its use as a famine food. The fruit and seed parts tested positive for all tests with health benefit endpoints while showing no obvious hazards indicating that further studies may consider them as functional foods, whereas the leaf tested positive in almost all assays/tests with health benefit but also hazard endpoints implying that further studies may consider it as herbal medicine for which the weight of benefit over risk needs to be verified to suggest any use, or otherwise restrict its use.

Evidences from epidemiological and experimental studies established an inverse relationship between consumption of some plant foods rich in antioxidant phytochemicals and the risk of several chronic diseases [76]. However, the translation of the epidemiologic relations of regular consumption of botanicals and preclinical data into clear results in clinical trials has been mostly uncertain. The "*antioxidant hypothesis*", which was initially used to justify the health benefits of phytochemicals, states that dietary antioxidants may directly neutralize free radicals in cells throughout the body, thereby protecting against diseases. However, this hypothesis was shown to have a clear problem. Currently, it is realized that many of the major phytochemicals, initially advertised as exerting health benefits by acting as free radical scavengers, instead act by inducing adaptive cellular stress responses. Recent reports focus on the "*hormesis hypothesis*" stating that at least some of the phytochemicals may prevent or mitigate various chronic diseases by activating adaptive stress response signaling pathways in cells [17,77]. Hormesis is a term used by toxicologists to refer to a biphasic dose response to an environmental agent characterized by a low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect. In the fields of biology and medicine hormesis is defined as an adaptive response of cells and organisms to a moderate (usually intermittent) stress. Examples include exercise, ischemic preconditioning, dietary energy restriction and exposure to low doses of certain phytochemicals [78]. The broad concept of adaptation in biology, overlaps with the more specific notion of hormesis in toxicology. An integrated biological system (an organism or a cell) upon interaction with a potentially damaging challenge, if not severely injured, may undergo a phenotypic shift, acquiring resistance to the same or a similar stressor. Adaptation operates *via* the stable activation of different mechanisms including repair to establish a new homeostatic condition. Maintenance of redox homeostasis (the endogenous capacity of cells to continuously deal with

challenges that generate electrophiles) by feedback mechanisms instead, is quite different from adaptation, but it fits similar criteria. Hormesis is, therefore, the term describing the seemingly positive adaptive responses to minimal exposure to agents that produce a well-defined damage when the exposure is higher [17,20]. More specifically, the term parahormesis has been used to describe the mechanism by which exogenous agents (nutritional antioxidants/phytochemicals) help maintain redox homeostasis by restoring the body's antioxidant defence [20]. The hormesis hypothesis suggests that cells throughout the body recognize some phytochemicals as potentially dangerous, and thus respond adaptively by engaging one or more stress signaling pathways that enhance the resistance of cells, organs, and the organism to a range of stressors that can cause or promulgate disease(s) [17,20,77]. Recent findings have elucidated the cellular signaling pathways and molecular mechanisms that mediate hormetic responses which typically involve enzymes such as kinases, and transcription factors such as Nrf2 and NF-κB. These feedback mechanisms operate at different level of complexity to achieve maintenance of redox homeostasis, and this hormesis/parahormesis seems to be the leading hypothesis to explain the mechanism by which botanicals and their phytochemicals provide health benefits [77,78]. The various beneficial endpoints identified in the present thesis also seem to fit into this hypothesis supporting possible health promoting potential of preparations or parts of *M. subcordata*. **Fig 4** summarizes the implications of in vitro data as obtained from the present work related to *M. subcordata* and suggests further research directions, considering both health benefits and hazards.

Moreover, despite numerous potential health benefits of botanicals, especially for these botanicals from wild collections such as *M. subcordata*, their safety issues should not be overlooked as they may pose health hazards due to their high content of some possibly toxic compounds. This implies that excessive and/or chronic consumption of such plant material may cause some toxicity problems and hence triggers the need for particular care with such species [79,80]. Besides, some constituents such as the isothiocyanates are known to deter people from consuming Brassica vegetables due to their sharp and bitter taste, despite their established beneficial health effects [81], which also holds true for some species of the caper family [82] including *M. subcordata*, which possess these natural products. This may require some process optimization [83] tailored at enhancing potential benefits and minimizing unwanted side effects. Finally, it should be noted that all data in the present study are from in vitro studies. Although in vitro systems have massively promoted our understanding of mechanisms of

biological events, numerous factors limit their extrapolation in to in vivo situation [84]. Thus, relevant in vivo studies may be warranted to validate the potential health benefits and/or hazards identified in this study. **Fig 5** outlines the framework by EFSA to assess the safety of a botanical or a botanical preparation as applied to *M. subcordata* showing data that has been obtained so far and that should be considered in further studies so as to help decisions on use or restriction of botanicals from this plant.

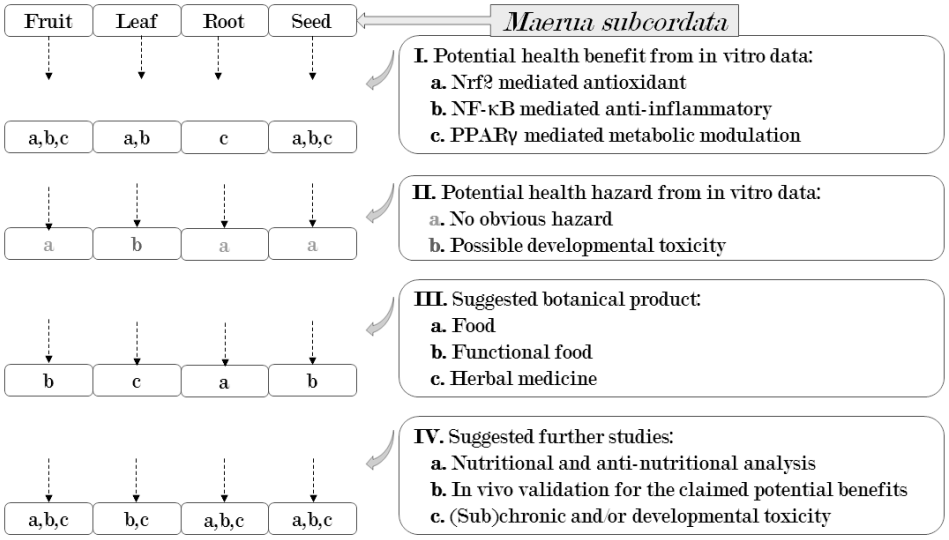

Figure 4. Showing results (I and II) of the present thesis applied to suggest possible botanical products (III) from *M. subcordata* and required further studies (IV).

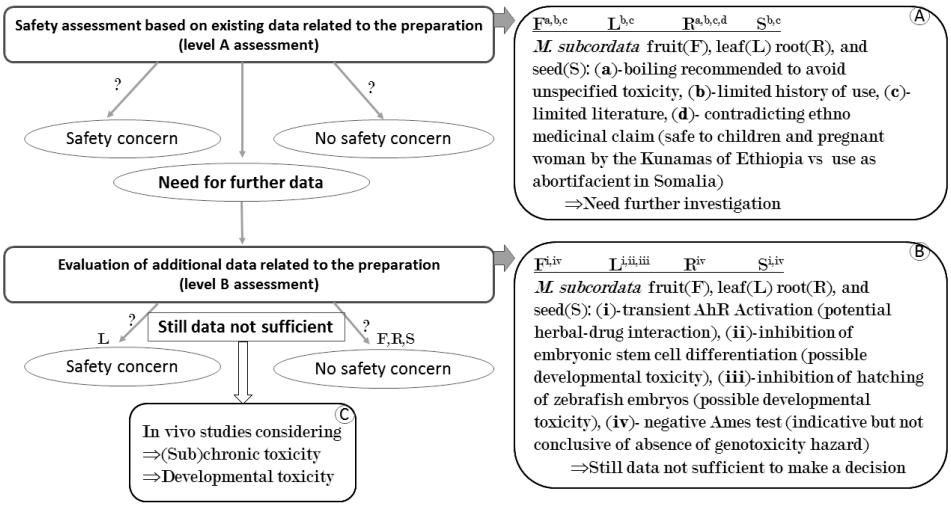

Figure 5. Showing the framework by EFSA to assess the safety of botanical products as applied to *M. subcordata* presenting what has been done (A and B) and suggesting what should be done in further studies (C).

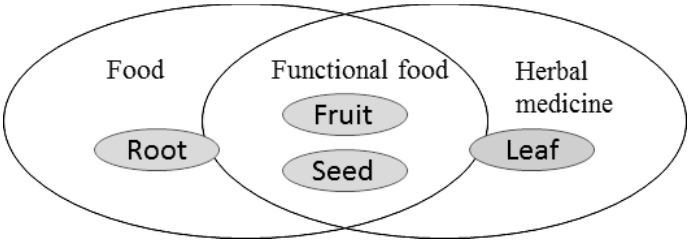

Figure 6: Graphical summary of potential research domains related to further studies on the fruit, leaf, root, and seed parts of *Maerua subcordata* based on the findings of the present thesis

Overall it is concluded that the results of the present thesis provided a proof of concept on how to evaluate possible dietary use of wild edible plants by using in vitro bioassays that help to consider potential hazards and benefits, thereby supporting the rational use of botanicals. The results obtained argue in favor of use of the root, fruit, seed and leaf parts of *M. subcordata* as (famine)food, functional food and/or herbal medicine, the exact application depending on the outcome of the benefit/hazard assessment performed in the present thesis (**Fig 5**).

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Chapter 7

Summary

Use of botanical products in the form of herbal supplements and herbal medicines continues to increase worldwide accompanied with increased expectations from consumers, regulators, and producers on quality and safety of these products. However, research on their quality and safety aspects has lagged behind, a circumstance that seeks industrial, academic, and governmental partnerships to address these research gaps. The present thesis provided a proof of concept on how to evaluate and contribute to support the rational use of botanicals, specially focusing on possible dietary use of wild edible plants, by using in vitro bioassays and considering potential hazards and benefits following consumption of botanical products.

Indigenous knowledge and traditional practices have core values in advancing our knowledge and understanding of cultural food-medicine systems, by providing a framework for scientific investigations that support the exploration of the health benefits and possible risks associated with the use of botanicals. Therefore, an ethnomedicinal and ethnobotanical study was provided in **chapter 2** of the present thesis that helped to plan relevant bioassays for the assessment of potential health benefits and possible hazards related to the use of botanical preparations or parts of *M subcordata*.

Botanicals contain numerous bioactive principles, called phytochemicals, that are responsible for the beneficial or toxic outcomes of the use of plant extracts. Also, previous phytochemical studies of *M subcordata* are limited. Thus, it was decided to perform profiling of candidate constituents of this plant using liquid chromatography coupled to mass spectroscopy followed by MAGMa software based structural annotation that resulted in the identification of some phytochemical groups like glucosinolates, quaternary ammonium compounds (betaines), and guanidine derivatives, among others. Along with the ethnobotanical data in **chapter 2** and literature data, this phytochemical overview helped the selection and framing of relevant bioassays to be applied in the experimental **chapters 3-5** of this thesis. This also helped to make possible associations of potentially responsible bioactive constituents to some of the endpoints obtained from these bioassays. Moreover, considering that enzyme hydrolysis with Viscozyme L is known to improve both extraction and biological activity of phenolic compounds and expecting that *M. subcordata* may contain such constituents, both enzyme hydrolysed (eh) and non-hydrolysed (nh) methanol extracts were prepared for screening in cell culture assays. Yet, the eh extracts did not show biological activities significantly different from the nh extracts in almost all assays tested. This may be because the major phytochemicals of this plant are

glucosinolates and biogenic amines rather than phenolic compounds and hence little affected by Viscozyme L based enzyme hydrolysis.

In **chapter 3** of this thesis, the potential health benefits of *M. subcordata* extracts through their effects on expression of EpRE-regulated genes, such as detoxifying phase II and antioxidant enzymes were described. The fruit, leaf, and seed extracts were shown to induce the expression of EpRE-mediated genes while induction by the root extract was minimal. Testing of selected candidates such as glucosinolates (or their matching isothiocyanates) and some biogenic amines showed that glucobrassicin and the isothiocyanates demonstrated strong induction while the biogenic amines exhibited either no significant induction or slight inhibition of EpRE-mediated gene expression. Stachydrine and trigonelline were among the biogenic amines that exhibited inhibition. Although both inducer and inhibitor candidate phytochemicals were identified in all extracts, an overall induction of EpRE mediated gene expression was displayed by fruit, leaf and seed extracts while no significant induction above solvent control was shown for the root extract. Since glucobrassicin was not detected in the root extract, it may be anticipated to contribute to the overall higher EpRE induction by the extracts in which it was detected. Hence, these data indicate that induction of EpRE-mediated transcription by *M. subcordata* candidate constituents may contribute to potential beneficial health effects associated to consumption of botanical preparations or parts of this plant.

C**hapter 4** of the present thesis describes the potential health benefits of *M. subcordata* extracts *via* their possible effect on the expression of NF-κB and PPARγ regulated genes as measured by their ability to inhibit LPS induced iNOS mediated nitric oxide production and to induce PPARγ-mediated gene expression, respectively. These effects have implications for anti-inflammatory effects and control of diseases of the metabolic syndrome. The fruit, root, and seed extracts were shown to induce PPARγ-mediated gene expression while induction by the leaf extract was minimal. On the other hand, all extracts inhibited LPS induced nitric oxide production, with the root showing the lowest potency. Testing of selected candidates such as isothiocyanates and some biogenic amines (e.g. stachydrine, trigonelline, agmatine) revealed inhibition of LPS induced nitric oxide production whereas no significant induction of PPARγ-mediated gene expression above solvent control was shown by the candidates for which definite identification was achieved. Thus, these data indicate that inhibition of NF-κB and induction of PPARγ-mediated transcriptions by *M. subcordata* candidate constituents may contribute to

possible beneficial health effects of anti-inflammatory activities and control of diseases of the metabolic syndrome associated to consumption of botanical preparations or parts of this plant.

In **Chapter 5**, a battery of in vitro tests was applied to identify selected endpoints that could be used to assess the potential hazards associated to consumption of botanical preparations or parts of *M. subcordata*. The Ames test and *in silico* (Derek Nexus) predictions were applied for initial genotoxicity screening whereas the aryl hydrocarbon receptor (AhR) and the estrogen receptor alpha (ERα) CALUX reporter gene assay as well as the embryonic stem cell test (EST) and the zebrafish embryotoxicity test (ZET) were applied to investigate alerts for developmental toxicity. In the initial *in silico* predictions some candidate compounds like isothiocyanates showed alerts for mutagenicity but in subsequent experiments, all extracts tested negative in the Ames test. A possible hazard of developmental toxicity was indicated for the leaf extract which tested positive for inhibition of embryonic stem cell differentiation in the EST and for inhibition of the hatching of zebrafish embryos in the ZET, whereas the other extracts (fruit, root, and seed) tested negative. Also, only the leaf extract tested positive for induction of ERα mediated gene expression but a definite conclusion on possible health benefit or risk of ERα activation requires consideration of several factors as ERα ligands are implicated in both beneficial health effects and potential endocrine disruption. All extracts, except the root, induced AhR mediated gene expression. This endpoint was aiming to assess the study plant for a possible dioxin like toxicity. However, unlike dioxins which cause persistent AhR activation that was related to the toxicological responses of the AhR, the induction by the extracts was transient, which is related to a physiological response of the AhR. Thus, this endpoint was not considered as an indicator of a hazard. Overall, the current data do not point to a genotoxicity hazard for *M. subcordata*. Yet, the data on the leaf extract may point towards a developmental toxicity hazard.

Chapter 6 summaries the outcomes of the experimental **chapter 3-5** and provides an overall discussions in the context of both potential health benefits and possible hazards. With regard to potential health benefits, the different endpoints from different bioassays carried out in the present thesis have been discussed in a holistic perspective elaborating their implications mainly in maintaining redox homeostasis. Emphasis was put on the potential capacity of *M. subcordata* extracts and phytochemicals therein, to differentially or co-ordinately interfere with pathologic oxidative and inflammatory stress pathways and thereby maintain redox homeostasis. Equally, safety concerns, especially in relation to the leaf extract, were addressed. Finally, an overall

conclusion and future perspectives related to further study and potential utilization of botanical preparations or parts of *M. subcordata* have been suggested. The results obtained argue in favor of use of the root, fruit, seed and leaf parts of *M. subcordata* as (famine)food, functional food and/or herbal medicine, the exact application depending on the outcome of the benefit/hazard assessment.

List of publications

1. Mebrahtom Gebrelibanos Hiben, Laura de Haan, Bert Spenkelink, Sebas Wesseling, Jochem Louisse, Jacques Vervoort, Ivonne M.C.M. Rietjens Effects of *Maerua subcordata* (Gilg) DeWolf on Electrophile-Responsive Element (EpRE)-Mediated Gene Expression *In vitro. PLoS ONE* 14(4): e0215155. https://doi.org/10.1371/journal.pone.0215155

2. Mebrahtom Gebrelibanos Hiben, Lenny Kamelia, Laura de Haan, Bert Spenkelink, Sebastiaan Wesseling, Jacques Vervoort, Ivonne M.C.M. Rietjens. Hazard Assessment of *Maerua subcordata* (Gilg) DeWolf. for Selected Endpoints using a Battery of *In Vitro* Tests. *Journal of Ethnopharmacology* 241 (2019) 111978.

3. Mebrahtom Gebrelibanos Hiben, Jochem Louisse, Laura de Haan, Ivonne M.C.M. Rietjens. Ethnomedicine and Ethnobotany of *Maerua subcordata* (Gilg) DeWolf (Submitted).

4. Mebrahtom Gebrelibanos Hiben, Laura de Haan, Bert Spenkelink, Sebastiaan Wesseling, Jacques Vervoort, Ivonne M.C.M. Rietjens. Induction of Peroxisome Proliferator Activated Receptor γ (PPARγ) Mediated Gene Expression and Inhibition of Induced Nitric Oxide Production by *Maerua subcordata* (Gilg) DeWolf. (Submitted).

5. Prinsloo G, Papadi G, Hiben MG, de Haan L, Louisse J, Beekmann K, Vervoort J, Rietjens IMCM. In vitro bioassays to evaluate beneficial and adverse health effects of botanicals: promises and pitfalls. *Drug Discov Today* 2017;22(8):1187-1200.

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Curriculum Vitae

Mebrahtom Gebrelibanos Hiben was born on February 24th,1978 in a village near a small town called Selekleka, Ethiopia where he was grown up and attended elementary school. He completed secondary education at Shire Senior Secondary School in 1998 and then continued higher education at Addis Ababa University, School of Pharmacy. He graduated in July, 2004 with B.Pharm degree and worked as line pharmacist at Addis Pharmaceutical Factory (Aug, 2004-Mar, 2005). Starting from March 2005, he got employed in Mekelle University, College of Health Sciences, School of Pharmacy. During his master study in pharmacognosy (2005-2007) at Addis Ababa University, School of Pharmacy, he studied the in vitro antioxidant properties of a local plant, *Senna singuana* (Del.) Lock. After graduation in September, 2007 with MSc degree in Pharmacognosy, Mebrahtom continued as academician and researcher at Mekelle University. Starting from July 2015 until October 2019, Mebrahtom worked as a PhD student on the project presented in this thesis, in the Division of Toxicology of Wageningen University. During this period, he completed the Postgraduate Education in Toxicology. After graduation in October, 2019 with PhD degree in Toxicology, Mebrahtom will continued as academician and researcher at Mekelle University.

Overview of completed training activities

Completed courses

Discipline specific activities

Food and Biorefinery Enzymology (Wageningen, 2015)

Cell Toxicology (Leiden, 2016)

Chemometrics (Wageningen, 2016)

Molecular Toxicology (Amsterdam, 2016)

Pathobiology (Utrecht, 2016)

Laboratory Animal Science (Utrecht, 2016)

Organ Toxicology (Nijmegen, 2017)

Ecotoxicology (Amsterdam/Wageningen, 2017)

Mutagenesis & Carcinogenesis (Leiden, 2019)

Toxicogenomics (Maastricht, 2019)

General courses

Data Management Planning (Wageningen, 2015)

Information Literacy PhD including EndNotes Introduction (Wageningen, 2015)

Philosophy and Ethics of Food Science and Technology (Wageningen, 2016)

Reviewing a Scientific Paper (Wageningen, 2016)

Scientific Publishing (Wageningen, 2016)

Risk assessment (Wageningen, 2016)

Towards a Global One Health (Wageningen, 2018)

Optional

Preparation of research proposal (2015)

PhD trip PhD trip division of Toxicology (Conference/Academic Exchange) (Japan, 2018)

Scientific presentations at Toxicology (2015-2019)

Food Toxicology (Wageningen, 2015)

Pharmacological Aspects of Nutrition (Wageningen, 2018)

General Toxicology (Wageningen, 2019)

Attended conferences/meetings

NVT Annual meeting, 2018 (Hilversum, The Netherlands, 2018)

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