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Central nervous system activities of extract *Mangifera indica* L.

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ABSTRACT

Ethnobotanical relevance: Leaves of *Mangifera indica* L. have folk-uses in tropical regions of the world as health teas, as a remedy for exhaustion and fatigue, as a vegetable, and as a medicine. *Mangifera indica* leaf extract (MLE) had previously been demonstrated to alter brain electrical activity in-vivo. The aim of the present series of studies was to investigate whether mangiferin, a major compound in leaves and in MLE, is responsible for the neurocognitive activity of MLE, and if the CNS activities of MLE have translational potential.

Materials and methods: MLE, tradename Zynamite, is produced by Nektium Pharma, Spain. Isolated mangiferin was tested in-vitro in radioligand binding and enzyme inhibition studies against 106 CNS targets. Changes in the electroencephalograms (EEG's) of MLE and mangiferin were recorded in-vivo from four brain regions. Two double blind randomized placebo-controlled crossover clinical trials were conducted, each with 16 subjects. At 90 min and at 60 min respectively, after oral intake of 500 mg MLE, EEG recordings, psychometric tests, mood state, and tolerability were studied.

Results: Isolated mangiferin is a selective inhibitor of catechol-O-methyltransferase (COMT) with an IC50 of 1.1 μM, with no activity on the CNS targets of caffeine. Both mangiferin and MLE induce similar changes in long-term potentiation (LTP) in the hippocampus in-vitro, and induce a similar pattern of EEG changes in-vivo. In both translational clinical trials MLE was well tolerated, with no cardiovascular side-effects. In both studies MLE caused significant spectral changes in brain electrical activity in cortical regions during cognitive challenges, different to the attenuated spectral changes induced by caffeine. There were no significant changes in the psychometric tests other than reaction time for all groups. In the second study there was a trend to faster reaction time within group for MLE ($p = 0.066$) and the percentage improvement in reaction time for MLE compared to placebo was significant ($p = 0.049$). In the first study MLE improved all scores for Profile of Mood States (POMS), with the score for "fatigue" significantly improved ($p = 0.015$); in the second study the POMS score for "dejection" was improved in the caffeine group, $p = 0.05$.

Conclusions: Mangiferin is a COMT inhibitor of moderate potency and is the major CNS-active compound in MLE. Both mangiferin and MLE increase hippocampal LTP in-vitro, and induce a similar pattern of changes in brain electrical activity in-vivo.

While the translational clinical trials of MLE are limited by being single dose studies in a small number of subjects, they provide the first clinical evidence that the extract is well tolerated with no cardiovascular side-effects, can induce changes in brain electrical activity, may give a faster reaction time, and decrease fatigue.

These CNS activities support the reported folk-uses use of mango leaf tea as a substitute for tea and as a traditional remedy for fatigue and exhaustion. Extract *Mangifera indica* L., Zynamite, has nootropic potential, and larger clinical studies are needed to realise this potential.

1. Introduction

Mangifera indica L., Anacardiaceae, is the well-known cultivated mango tree of the tropical and sub-tropical regions of the world. The fruits are seasonal and are used as food at almost every stage of development. The pulp of young sour varieties is sun-dried and powdered for use as seasoning for the preparation of Indian foods, older raw green fruit is processed into spicy relishes eaten with curries, and the peeled ripe fruit is eaten as a dessert or as a delicious whole fruit.

While not as widely known as the fruit, the leaves of *Mangifera*

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indica have widespread use as tea, food, and medicine. Over a century ago, Chinese traders in the Philippines used mango leaves as a substitute for tea, as they are "similar in colour, taste, aroma and tonic properties" (MacMicking, 2007). The young leaves are consumed as a vegetable in India, Myanmar, Cambodia, Indonesia, Philippines, the Pacific Islands (Bally, 2006; Budhwar, 2002; Facciola, 1990; Khaing, 1978; Martin et al 1975, 1998; Rajyalakshmi, 2002). Infusions of the leaves are taken as a health tea in India and Nigeria and for treating fatigue, exhaustion, pain, fever, stroke, diarrhoea, dysentery, typhoid fever, oedema, sore throat and scurvy (Doughari and Manzara, 2008; Fowler, 2006; Idu and Onyibe, 2007; Campbell et al., 2002). In Nigeria the leaf decoction is used for treating hypertension and malaria (Igoli et al., 2005; Odugbemi et al., 2006). Indigenous people in South America have been reported to combine mango leaves with the leaves of yerba maté as a tea (Campbell, 1996).

Extracts of *Mangifera indica* leaves have been shown to have a wide range of pharmacological activities in-vitro and in-vivo, including antioxidant, anti-inflammatory, antibacterial, antifungal, antiplasmodial, analgesic, neuroprotective, hepatoprotective, immunomodulatory, antihyperlipidaemic, antidiabetic and gastroprotective (Ediriweera et al., 2017). The standardized extract of *Mangifera indica* leaves, Zynamite (MLE), has been shown to improve sports performance in humans in combination with quercetin or luteolin (Gelabert-Rebato et al., 2019a, 2019b, 2018; Martin-Rinco et al., 2020). MLE has been demonstrated to alter brain electrical activity in a similar way to caffeine when given by gavage in an in-vivo model, and to increase hippocampal LTP (Dimpfel et al., 2018). It was concluded that bioactive compounds from this extract are absorbed and cross the blood brain barrier. Mangiferin, a xanthone polyphenol (see Fig. 1), and the major compound in MLE, was thought to be the compound most likely to be responsible for CNS activities.

The concentration of mangiferin in *Mangifera indica* varies according to the part of the plant, the plant variety, and the age of the leaves (Das et al., 2012; Duang et al., 2011; Kaivalya et al., 2011; Lei et al., 2012; Rodeiro et al., 2014). The highest concentration of mangiferin is present in leaves (Das et al., 2011) at 36.9 g/kg dry weight mangiferin in old leaves and 58.12 g/kg in young leaves (Barreto et al., 2008). The lowest concentration of mangiferin is present in root, seed or pulp, at up to 2.65 mg/kg dry weight (Hewavitharana et al., 2013). Levels in skin of the fruit are 4.94 g/kg dry weight (Das et al., 2012; Dou et al., 2014; Louisa et al., 2014; Lv et al., 2013; Pan et al., 2014; Rajendran et al., 2014) and in bark 18.33 g/kg dry weight (Barreto et al., 2008).

Mangiferin has a wide range of biological activities in in-vitro and in-vivo studies, including antibacterial, antifungal, antiviral, antioxidant, analgesic, anti-inflammatory, antidiabetic, anticancer, gastroprotective, cardioprotective, hypolipidaemic, neuroprotectant and antiallergic activities (Sekar, 2015). Diverse CNS effects have been reported for mangiferin including improved long-term cholinergic memory deficits by inhibition of acetylcholinesterase or by cholinergic receptor stimulation and inhibition of NF-κB activation (Jung et al., 2009), MAO_A and MAO_B inhibition and antidepressant-like effects (Dimitrov et al., 2011), significantly improved learning and memory in a scopolamine model of aging (Biradar et al., 2012; Sethiya and Mishra, 2014), attenuated dopaminergic neurodegeneration in-vivo (Kavitha et al., 2013). Mangiferin was shown to be neuroprotective (Sekar, 2015) and prevented the cognitive deficits and hippocampal BDNF depletion induced by $AlCl_3$ (Kasbe et al., 2015), ameliorated cognitive deficits induced by lipopolysaccharide (LPS), and decreased LPS-induced IL-6 production in the hippocampus (Fu et al., 2015), reduced TAU hyperphosphorylation in the cortex and hippocampus, reduced inflammation, and improved episodic and spatial memory. (Infante-Garcia et al., 2017). Mangiferin ameliorated stress-induced behavioural abnormalities and the down-regulation of the expression of NLRP3, the adaptor protein ASC, and caspase-1, which subsequently reduced the production of IL-1β and IL-18, indicating that mangiferin exerts antidepressant-like effects in this model (Cao et al., 2017).

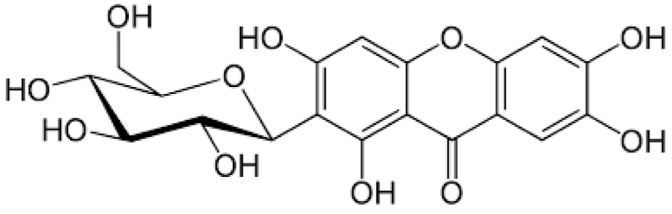


Fig. 1. Structure of mangiferin.

Recent randomised controlled clinical trials have shown positive effects of MLE in combinations with either quercetin or luteolin on sports performance, including increased mean power output, peak power output, brain oxygenation, VO_2 max, and muscle oxygen extraction; and decreased lactate production. These activities are thought to be partly based on both central and peripheral activities of mangiferin, including free radical-scavenging, inducing the antioxidant gene program, and down-regulation of the expression of superoxide-producing enzymes (Gelabert-Rebato et al., 2019a, 2019b, 2018; Martin-Rinco et al., 2020).

MLE has been studied for safety in a 90-day repeat dose study in rats. The No Observed Adverse Effect Level (NOAEL) of this extract was 2000 mg/kg body weight per day by gavage, the highest dose studied (Reddeman et al., 2019), and the extract has self-affirmed Generally Recognized As Safe (GRAS) regulatory status in the United States, where it is sold as a dietary ingredient in the food, beverage, and dietary supplement industries.

The purpose of the present series of studies was to investigate:

1. If mangiferin, the major compound in MLE, causes similar changes in brain electrical activity in the hippocampal slice model reported for MLE (Dimpfel et al., 2018).
2. If mangiferin has in-vitro binding activities on the same molecular targets as caffeine, namely antagonist activity on the adenosine receptors and inhibition of PDE4.
3. If mangiferin causes similar changes in brain electrical activity in-vivo as reported for MLE (Dimpfel et al., 2018).
4. If the reported in-vivo brain activating activity of MLE (Dimpfel et al., 2018) has translational potential in pilot psychophysiological clinical studies

2. Material and methods

2.1. Material

MLE is a proprietary *Mangifera indica* leaf extract standardized to 60% mangiferin, produced by Nektium Pharma S.L. in Spain. Mango leaves from commercial mango fruit trees were harvested and air-dried before milling, maceration in water, concentration, and spray-drying. The species of the leaves was confirmed to be *Mangifera indica* L. by DNA fingerprinting by Real Jardín Botánico, CSIC, Spain. *Mangifera indica* L. is an accepted name contained in the plant list database (http://theplantlist.org; accession date 12/10/2019).

The leaf extract was concentrated and spray dried resulting in a plant:extract ratio of 15–30:1 by weight for the final dry extract. The extract was confirmed by HPLC analysis to contain no caffeine, theobromine, theacrine, or theophylline. Extraction yield is estimated based on the content of mangiferin in both the mango leaves used as raw material, and the final extract. The production process, which mainly consists of water extraction at high temperature, purification by affinity resin columns, concentration and spray drying, provides recovery yields above 90% for mangiferin (w/w).

The mangiferin reference compound was sourced from Indofine Chemical Company, Inc. (USA), lot number 0910077. Caffeine was

sourced from Sigma-Aldrich (Switzerland), lot number BCBK0578V.

2.2. In-vitro studies of MLE and mangiferin

2.2.1. Hippocampal slice long-term potentiation

Hippocampal slice preparation and stimulation is a validated model for direct investigation of the interaction of test substances with living neuronal tissue (Dingledine, 1984; Lynch and Schubert, 1980). Since the three-dimensional structure of the hippocampal tissue is preserved in the slices, effects of investigational substances on the excitability of hippocampal pyramidal cells can be studied. Direct electrical stimulation of Schaffer Collaterals with single stimuli or theta burst stimuli leads to release of glutamate, resulting in excitation of postsynaptic pyramidal cells. The result of the electrical stimulation is recorded as a population spike representing the number of recruited pyramidal cells (Dimpfel et al., 2016). This model was used for example to demonstrate that the pharmaceutical memantine, used in the treatment of dementia, can increase the population spike amplitude in response to both single stimuli and theta burst stimulation, and thus increase hippocampal long-term potentiation (Dimpfel, 1996).

In a previous study, 25 mg/ml of MLE changed excitability of the hippocampus of rats in an in-vivo model (Dimpfel et al., 2018). To evaluate if mangiferin is responsible for the stimulating effect of MLE, isolated mangiferin was tested in a hippocampal slice preparation assay and compared to the effect of MLE in-vitro.

Hippocampus slices were obtained from 21 male Sprague Dawley rats (Charles River Wiga, Sulzbach, Germany). The animal studies were performed in line with the European Community guidelines "on the protection of animals used for scientific purposes" (EEC Directive of 1986; 2010/63/EU). Rats were kept under a reversed day/night cycle for 1 week prior to the start of the experiments. Animals were exsanguinated under ether anesthesia; the brain was removed in total and the hippocampal formation was isolated under microstereoscopic sight. The midsection of the hippocampus was fixed to the table of a vibrating microtome (Rhema Labortechnik, Hofheim, Germany) using a cyanoacrylate adhesive, submerged in chilled bicarbonate-buffered saline (artificial cerebrospinal fluid (ACSF)): NaCl: 124 mM, KCl: 5 mM, CaCl2: 2 mM, MgSO4: 2 mM, NaHCO3: 26 mM, glucose: 10 mM, and cut into slices of 400 µm thickness. All slices were pre-incubated for at least 1 h in Carbogen saturated ACSF (pH 7.4) in a pre-chamber before use (Dimpfel et al., 1991). The respective mg/L treatment doses of pure mangiferin were 0.05, 0.10, 0.40, 0.70 and 1.0 mg/l, and of MLE were 0.1, 0.3, 0.5, 0.75 and 1.0 mg/L.

During the experiment the slices were held and treated in a special superfusion chamber (List Electronics, Darmstadt, Germany) at 35 °C. The preparation was superfused with ACSF at 180-230 ml/h. Electrical stimulation (200 mA constant current pulses of 200 ms pulse width) of the Schaffer Collaterals within the CA2 area and recording of extracellular field potentials from the pyramidal cell layer of CA1 (Dimpfel et al., 1991) was performed using the “Labteam” Computer system “NeuroTool” software package (MediSyst GmbH, Linden, Germany). Measurements were performed at 10 min intervals to avoid potentiating mechanisms. Four stimulations – each 20s apart – were averaged for each time point. After obtaining three stable responses to single stimuli (SS), LTP was induced by applying a theta burst type pattern (theta burst stimuli, TBS). The mean amplitudes of three signals were averaged to give the mean of absolute voltage values (Microvolt ± standard error of the mean) for four slices representing one of the experimental conditions. Four slices were used from 1 rat per day. Either 2 or 4 slices were averaged to give one value (i.e. one concentration). During this pilot screening, control values were taken from an earlier study (n = 12 slices). For statistical purposes the non-parametric Wilcoxon test and Mann-Whitney *U*-test was used.

2.2.2. In-vitro studies of mangiferin on CNS targets

Caffeine causes most of its biological effects by antagonizing adenosine receptors (ARs) and by inhibiting phosphodiesterases (PDEs) (Ribeiro and Sebastião, 2010). To investigate if mangiferin, the major bioactive in MLE, shared the same brain targets as caffeine, 106 in-vitro radioligand binding assays were conducted (68 receptors and transporters, 24 enzymes and 14 PDEs). The broad screen of the activity of mangiferin on multiple CNS targets was undertaken in order to identify additional CNS targets for mangiferin.

2.2.2.1. Radioligand binding assay. A concentration of 1.0E-05M of mangiferin was tested against a panel of 68 receptors (radioligand binding assay, Eurofins Cerep SA, Le Boisl Évêque, France): Adenosine A_1, A_2, A_3, $\alpha_1$adrenoceptors (non-selective); $\alpha_2$adrenoceptors (non-selective); $\beta_1$adrenoceptors, $\beta_2$adrenoceptors, angiotensin AT1 receptors, AT2 receptors, benzodiazepine (BZD) binding sites (central); bradykinin B1 receptors, B2 receptor, cannabinoid CB1 receptors, CB2 receptors, cholecystokinin CCKA (CCK_1) receptors, CCKB (CCK_2) receptors, Corticotropin-releasing hormone receptor 1 (CRF1), dopamine D1 receptors, D2S receptors, D3 receptors, D4.4 receptors, endothelin ETA receptors, ETB receptors, GABA receptors (non-selective), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), Kainate, NMDA receptors (phencyclidine PCP binding site), histamine H1 receptors, H2 receptors, H3 receptors, Imidazoline 2 (I2), Cysteinyl leukotriene-CysLT1 (LTD4), melanocortin MC_4receptors, melatonin MT1 receptors, muscarinic cholinoceptors M1 receptors, Neurokinin NK1 receptors, NK2 receptors, NK3 receptors, neuropeptide Y receptors, N neuronal nicotinic receptor α4β2, opioid receptors, orexin ORL1 receptors (NOP), Peroxysomal proliferator activated receptors gamma ($PPAR\gamma$), Phencyclidine (PCP), Prostaglandin 2 (EP_2), ATP P2X receptors, P2Y receptors, sigma receptors, glucocorticoid Receptors (GR), oestrogen receptor (ER), progesterone (PR), androgen receptor (AR), Thyrotropin-releasing hormone 1 (TRH_1), vasopressin V_{1a} receptors, V_2 receptors, Ca^{2+}channel (L, dihydropyridine site) (phenylalkylamines), Ca^{2+}channel (L, diltiazem site) (phenylalkylamines), Ca^{2+}channel (L, verapamil site) (phenylalkylamines), K_{ATP} Channel, K_V channel, SK_{Ca} channel, Cl^- channel (GABA-gated), noradrenaline transporter, dopamine transporter, GABA transporter, Choline transport (CHT1), 5-HT transporter, and Serotonin (5-hydroxytryptamine) 5-HT1 (non-selective).

2.2.2.2. Cellular and nuclear receptor functional assay. A concentration of 1.0E-05M of mangiferin was tested to evaluate the percentage of agonist and/or antagonist response against the Transient Receptor Potential Vanilloid 1 (TRPV1) (Eurofins Cerep SA, Le Boisl Évêque, France). The references compounds used were capsaicin as agonist and capsazepin as antagonist.

2.2.2.3. Enzyme and uptake assays. A concentration of 1.0E-05M of mangiferin was tested to evaluate the percentage of inhibition of a panel of 24 enzymes (Eurofins Cerep SA, Le Boisl Évêque, France): Cyclooxygenase 1 (COX 1), 5-lipoxygenase, Phosphodiesterases PDE1B, PDE3A, PDE4D2, and PDE5 (non-selective); Phosphatase 1B (PTP1B), phosphatase CDC25A, Protein kinase C alpha (PKCα), acetylcholinesterase, Catechol-O-Methyl transferase (COMT), GABA transaminase, monoamine oxidase-A (MAO-A, monoamine oxidase-B (MAO-B) recombinant enzyme, tyrosine hydroxylase, ATPase (NA^+/K^+), Centromere-associated protein-E (CENP-E), Eg5 (kinesin-5 protein member), histone deacetylases HDAC3, HDAC4, HDAC6, and HDAC11; sirtuin 1, and sirtuin 2 (inhibitor effects).

2.2.2.4. Phosphodiesterase (PDE) activity assays: Enzyme and uptake assays. A concentration of 1.0E-05M of mangiferin was tested to evaluate the percentage of inhibition of a panel of 14 enzymes (Eurofins Cerep SA, Le Boisl Évêque, France). The following PDE enzymes (human recombinant expressed in Sf9cells) were used along with corresponding reference compounds: PDE1B with Nitrendipine,

PDE2A2 with EHNA, PDE3A and PDE3B with milrinone, PDE4A1A, PDE4B1 and PDE4D2 with Ro20-1724, PDE7A1 with BRL 50481, PDE8A1 with trequinsin, PDE10A2 with papaverine, PDE11A with dipyridamole, AMPKα with staurosporine. In the following, PDE5 (human platelets) and PDE6 (non-selective) (bovine retina) were used along with dipyridamole and zaprinast as reference comounds respectively.

In most assays, production of [3H]-5'AMP from [3H] cAMP was measured by scintillation counting after 20 min at room temperature. The PDE1B production of [3H]-5'-cGMP from [3H]-cGMP was measured by scintillation counting after 20 min at room temperature. The PDE5 and PDE6 production of [3H]-5'-cGMP from [3H]-cGMP was measured by scintillation counting after 60 min at room temperature. AMPKα production of phosphor-Ulight-CREB from ATP was measured by LANCE© after 30 min at room temperature.

2.2.2.5. COMT (catechol-O-methyl transferase): Enzyme and uptake assays. A specific catechol-O-methyl transferase (COMT) assay was performed to determine IC50 and EC50 of pure mangiferin (Eurofins Cerep SA, Le Boisl Évêque, France). COMT (Catechol-O-methyl transferase) from porcine liver was used and the reference compound used was Ro 41-0960 with an IC50 (M) of 8.4E-08M. Production of scopoletin from esculetin was measured by fluorimetry counting after 30 min at 37 °C. A range of MLE concentrations (1.0E-05M, 1.0E-06M, 3.0E-07M, 1.0E-07M, 3.0E-08M, 1.0E-8M, 3.0E-09M, 3.0E-10M) were tested on COMT.

2.2.2.6. Statistics. In the binding assays, enzyme assays and uptake assays, results are expressed as a percentage of control specific binding ((measured specific binding/control specific binding) × 100) obtained in the presence of test material and as percentage inhibition of control specific binding ((100-(measured specific binding/control specific binding) × 100) obtained in the presence of test materials. In cellular and nuclear receptor functional assays, the results are expressed as percentage of control agonist response or inverse agonist response ((measured response/control response) x 100) and as percentage inhibition of control agonist response ((100-(measured response/control response) x 100) obtained in the presence of test material.

Results showing an inhibition or stimulation higher than 70%, were considered a “hit”, representing a potential physiological effect of the test compound on that target, and which then warranted proceeding with dose-response testing. The IC50 values (concentration causing a half-maximal inhibition of control specific binding), EC50 values (concentration producing a half-maximal increase in control basal activity) and Hill coefficients (nH) were determined by non-linear regression analysis of the inhibition/concentration-response curves generated with mean replicate values using Hill equation curve fitting ($Y = D + [(A - D)/(1 + (C/C50)nH)]$, where Y is the specific binding, D the minimum specific binding, A the maximum specific binding, C the compound concentration, C50 = IC50, and nH is the slope factor). The inhibition constants (Ki) (for binding assays) were calculated using the Cheng Prusoff equation ($Ki = IC50/(1 + L/K_D)$), where L is concentration of radioligand in the assay and K_D is affinity of the radioligand for the receptor. A scatchard plot is used to determinate the K_D. For the antagonist, the apparent dissociation constants (K_B) (in cellular and nuclear receptor functional assays) were calculated using the modified Cheng Prusoff equation ($K_B = IC50/(1 + A/EC50A)$), where A is the concentration of reference agonist in the assay and EC50A = EC50 the value of the reference agonist.

All analyses were performed using Hill software developed at Eurofins Cerep SA, Le Boisl Évêque, France, and validated by comparison with data generated by the commercial software® 4.0 for Windows® (©1997 by SPSS Inc.)

2.3. Changes in brain electrical activity in-vivo by quantitative EEG

To characterize the neurophysiological action of mangiferin, and compare it to the effect of MLE, quantitative electroencephalography (qEEG) was recorded wirelessly from freely moving rats as described earlier (Dimpfel, 2003). Eight Fischer rats were implanted with a set containing 4 bipolar concentric steel electrodes and a transmitter that sent local electrical field potentials wirelessly to a computer for frequency analysis. Transmitted data are processed by Fast Fourier Transformation (FFT) and spectral power documented for 8 frequency ranges (delta, theta, alpha1, alpha2, beta1a (beta a), beta 1b (beta b), beta2 and gamma) within frontal cortex, hippocampus, striatum and midbrain reticular formation at hourly intervals.

A crossover design with at least one week of wash-out between administrations was used. The control consisted of gavage administration of 1 ml/kg of the 0.9% NaCl vehicle. The test substances were 50.0 mg/kg MLE (Batch No. MLF01-170201) and 25 mg/kg of pure mangiferin. After a pre-treatment period of 45 min for recording a time-averaged baseline, treatment effects were observed continuously on the screen (artifact control) for 300 min subdivided into 15 min periods, after a delay of 5 min for calming of animals after gavage administration. Changes of electric power (μV) are expressed as a % of the 45 min pre-treatment spectral power values within each frequency band. Data were averaged from 8 animals.

Effects of the treatments on the motion of the animals were recorded by video vigilance for the entire duration of the experiment. Changes in motion (cm/h) were given for the entire 5 h after treatment administration. Mean average values are given S.E.M. (Standard Error Mean) Statistical comparison of the results of MLE, mangiferin and control were determined using Wilcoxon test and Mann-Whitney *U*-test (p values are given on the right side).

2.4. Psychophysiological effects in pilot translational clinical studies

To investigate whether the reported CNS activating activities of MLE shown earlier (Dimpfel et al., 2018) have translational potential, two pilot double-blind, randomized, placebo-controlled, cross–over, clinical trials were conducted in healthy subjects. The effect of a single dose of 500 mg MLE (containing 300 mg mangiferin; Batch No. MLF01-170201) was evaluated in 2 separate studies, respectively at 90 min after intake (study 1) and at 60 min after intake (study 2).

2.4.1. Objectives

The main objective was to evaluate the psychophysiological effects of a single dose of 500 mg MLE versus placebo (study 1) and 500 mg MLE versus placebo and versus 160 mg caffeine (study 2) in humans based on three levels of evidence: qEEG recordings, psychometric tests and questionnaires after administration of a single dose. The tolerability of the extract was assessed in both, study 1 and study 2. Changes in heart-rate variability and blood-pressure were evaluated in study 1.

2.4.2. Subjects and inclusion/exclusion criteria

16 healthy male and female subjects (7 males and 9 females in study 1, and 6 males and 10 females in study 2), were recruited by advertisement for each study and invited to participate. Participants were German-speaking, age 18–40, right-handed and with unremarkable medical history or clinical findings. To be included in study 2, the participants had to score higher than 7 in a concentration questionnaire and report average consumption of 1-3 cups of coffee daily (about 75-225 mg caffeine/day). All participants signed an informed consent form and were advised not to drink caffeinated drinks 18 h before the trial and no alcohol at least 12 h before attending the examination days (proof of alcohol was performed on the examination days).

Exclusion criteria included a history of acute or chronic diseases, psychiatric disorders (anamnestic survey), clinically relevant allergies, intake of clinically relevant medication in the previous 2 weeks, known

intolerance, hypersensitivity or allergies to herbal extracts or to any of the other ingredients of the investigational products.

Both studies were performed in accordance with the current version of the Declaration of Helsinki (Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. 52nd WMA General assembly, Edinburgh, Scotland. 2000). Both, study 1 (case number FF90/2017) and study 2 (case number FF26/2018), were conducted in agreement with the International Conference on Harmonization (ICH) guidelines on Good Clinical Practice (GCP) and were approved by the ethics committee, Landesärztekammer Hessen, ImVogelsgesang 3, D-60488 Frankfurt, Germany.

2.4.3. Study designs

Both studies followed a similar protocol design and were performed by NeuroCode AG, Sportparkstr. 9, D-35578 Wetzlar, Germany. In study 1 (internal trial number NCAG2517), the effect of a single oral dose of 500 mg of MLE was compared to placebo 90 min after intake. In study 2 (internal trial number NCAG1218), the time after intake was reduced and the effect of MLE was compared to placebo, to caffeine and to a combination of caffeine plus MLE 60 min after intake. Both studies were randomized, double-blind, placebo-controlled trials with cross-over design, conducted in 16 healthy adult subjects, without statistical difference between gender.

In both studies the primary outcome measures were changes in regional electric brain activity quantified as spectral power during psychometric tests. Secondary outcome measures in both studies were changes in the psychometric tests: the d2 test (d2) for attention, the calculation performance test (CPT), the memory test (ME), the reaction time test (RT-Test), the number sequence test (NST) (the latter only in study 1), and the number connection test (NCT). In the second study, latency and amplitude of acoustically and visually evoked P300 were measured. P300 is an electrical signal evoked by acoustic or visual stimulant used as neurophysiological parameter related to cognition (Polich J 1995). The RT-test was performed without parallel recording of the brain activity. As additional secondary outcomes for both studies, changes in Profile of Mood States (POMS) questionnaire, tolerability, heart rate variability (study 1), heart rate (study 2), and blood pressure were evaluated.

The qEEG was recorded as previously described (Dimpfel, 2003; Dimpfel et al., 2011). The qEEG recorded the brain electrical activity in 17 different brain regions (17 scalp surface electrodes according to the international 10/20-system with Cz as physical reference electrode (Computer aided topographical electroencephalometry: CATEEM® using an electro cap) within the 6 defined frequency ranges (delta, theta, alpha1, alpha2, beta1 and beta2). Baseline recording (first recording) for 6 min under the condition of eyes open (EO) was followed by the psychometric tests as shown in Fig. 2. qEEG data were recorded twice: before (baseline, both studies) and at 90 min (study 1) or 60 min (study 2) after the intake of the supplement. Between the measurements, subjects spent their time in the facility's recreation room. All experiments took place at the same time of the day (starting at 07:00). Data were analyzed from 1.25 to 35 Hz using the CATEEM® software. In order to compare the efficacy of MLE and other products, two EEG brain regions of interest (ROI) were defined: frontal cortex (average of electrode positions Fz, F7 and F8) and association cortex (average of electrode positions P3, Pz, P4, T5 and T6). These two brain areas have been shown to be responsible for higher cognitive functions and changed their frequency content during cognitive testing in earlier experiments. By setting the absolute spectral power during baseline recording to 100%, preparation induced changes can be documented as % change from baseline. Thus, in the presence of placebo no major changes should emerge.

P300 was measured only in study 2. Subjects were asked to respond by counting the rare target stimuli. Event-related potentials (ERP) to both rare and frequent stimuli were recorded from all 17 scalp electrodes. The sampling rate was 2,048 Hz. Sampling began 100 ms before stimulus onset and continued until 500 ms post-stimulus. Stimulus presentation, artifact rejection and averaging of waveforms were performed online by CATERPA software. Event related potentials (ERP) elicited by rate and frequent stimuli were averaged separately following a rejection of artifact-containing epochs (> 30% of EOG-related maximum amplitude). Prior to the averaging, single trial ERP data were digitally filtered with a band-pass of 0.2 - 330 Hz. Differences between the wave forms, elicited by the standard and target stimuli were calculated and the P300 peak amplitudes and latencies were determined using a mathematical peak detection paradigm (only the P300 amplitude at electrode position Pz was considered). Subjects were tested sitting in an upright position. They were instructed to keep their eyes open and to fix a defined point 1.5 m in front of them.

Validated psychometric tests were performed before and after intake of the trial preparations. A total of 6 psychometric tests were performed in the presence of qEEG recording: concentration d2, ME, CPT, RT, and NCT. A NST was used in study 1 (Düker and Lienert, 1965; Merten, 1997).

The POMS questionnaire was filled out before intake of the trial preparation at 90 min (study 1) and at 60 min (study 2) after intake. It assesses transient, distinct mood states. The POMS is a standard validated psychological test formula (Grulke et al., 2006). The questionnaire contains 65 words/statements that describe feelings people have. The test is required to indicate for each word or statement how one has been feeling in the past week including today. Score 1: "dejection"; Score 2: "sullenness"; Score 3: "fatigue"; Score 4: "thirst for action".

2.4.4. Statistical analysis

qEEG data from the first recording session before the intake of the investigational capsules are calculated as absolute numbers (μV2). For statistical evaluation the non-parametric sign test was used. For mathematical differentiation of the different mental loads the linear discriminant analysis according to Fischer was used. In order to document statistically the different electric reactions of the brain to various cognitive loads, data from each challenge were documented as absolute spectral power (μV2). Comparison of MLE versus placebo was accomplished by evaluation of the second recording of the day, 90 min (study 1) or 60 min (study 2) after intake in comparison to the baseline values. Data from the first recording (baseline) were set to 100% and electrophysiological changes produced by placebo or the extract were depicted as %-changes thereof. The non-parametric sign test is chosen for comparison between placebo and MLE. Exploratory statistics give p values, which are presented at the appropriate site.

3. Results

3.1. In-vitro studies of MLE and mangiferin

3.1.1. Hippocampal slice model

The presence of both isolated MLE and Mangiferin led to a similar pattern and amplitude of time dependent increases of the population spike amplitude in comparison to the control (Fig. 3a and 3b) during both single shock stimulation (SS) and during theta burst stimulation (TBS) (numerical data presented in Table 1).

The presence of 1 mg/L of MLE (Fig. 4a) and 0.7 mg/L of pure mangiferin (Fig. 4b) in the hippocampus slice-preparations led to greater time dependent increases of the population spike amplitude in comparison to control for single stimuli, and to greater LTP for theta burst stimuli. The pattern of changes of population spike amplitude and long-term potentiation was similar to both MLE and mangiferin.

3.1.2. In-vitro studies of mangiferin on CNS targets

To analyse whether isolated mangiferin has in-vitro binding or inhibitory activities on the same molecular targets as caffeine, namely

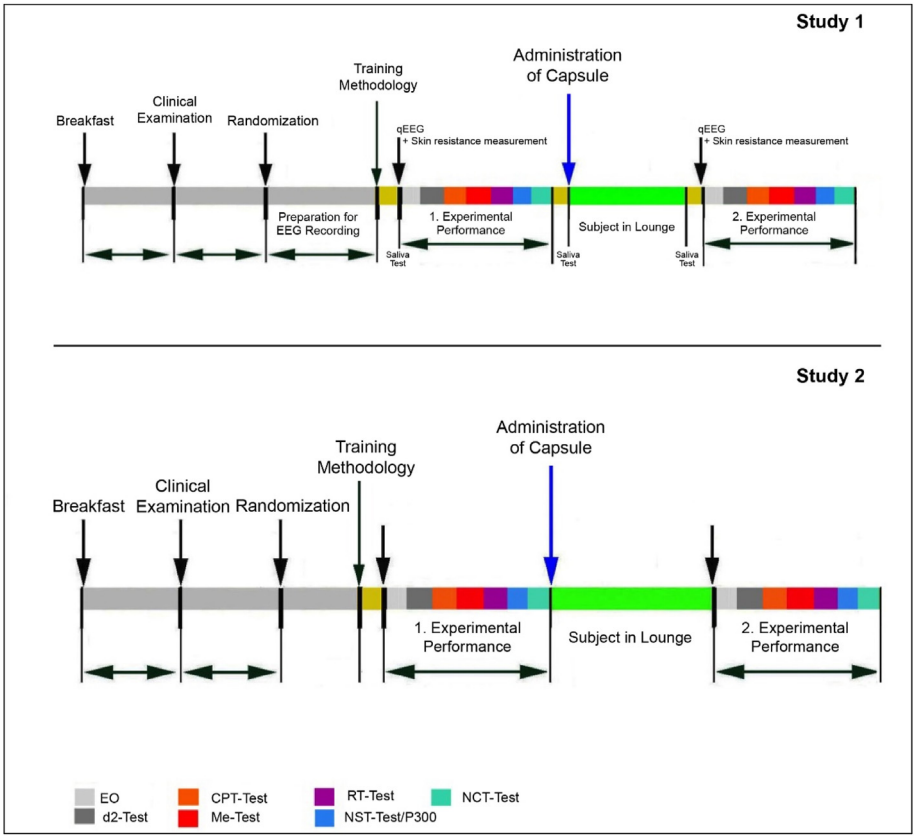


Fig. 2. Timeline of the experimental protocol of study 1 and study 2. Performance: Eyes open (EO) and different cognitive tests (d2-test, calculation performance test (CPT-Test), memory test (ME-Test), number connection test (NCT-Test), number sequence test (NST-Test), reaction time test (RT-Test).

adenosine receptors and PDE4, or acts on other CNS targets, pure mangiferin was screened via binding assay at 1.0E-05M (4.23 1 μg/ml) on a panel of 93 CNS targets. No inhibition (or stimulation) for assays run in basal conditions was higher than the previously defined threshold for a "hit" on a target, a value greater than 70%, was observed for any target with exception of catechol-O-methyltransferase (COMT) (Figs. 5 and 6). COMT showed a marked effect with inhibition of 97.7% (Fig. 7A). Based on this initial "hit", an in-vitro assay was performed against COMT at different concentrations to determine IC50 of pure mangiferin (Fig. 7B) and the result was an IC50 of 2.6E-06M, equivalent to 1.1 μg/ml.

3.2. Changes in brain electrical activity in-vivo by qEEG

Previously, changes in brain electrical activity in rats determined by qEEG had been demonstrated for MLE (25 mg/ml) compared with caffeine (Dimpfel et al., 2018), and here the effect of 50 mg/kg of MLE on changes of spectral power in rats by qEEG was compared with the effect of 25 mg/kg pure mangiferin.

The saline vehicle resulted in no changes of qEEG spectral power

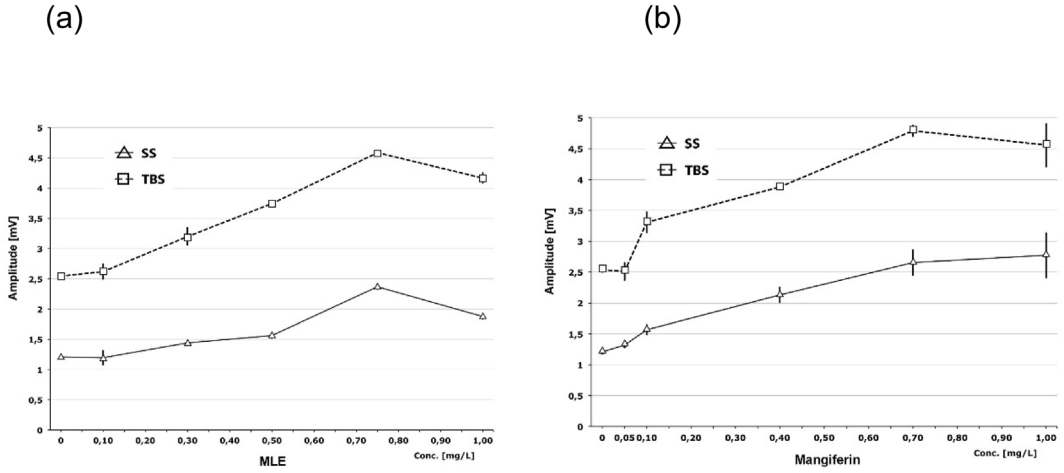


Fig. 3. Concentration dependent effects of MLE (a) and pure mangiferin (b) on pyramidal cell activity in terms of changes of population spike amplitudes (as voltage on the ordinate) during single stimuli (SS) as well as during theta burst stimuli (TBS). Results as obtained after performance of single stimuli (10-80 min) or after burst stimuli (90-120 min). Data are given as mean ± S.E.M.

Table 1
Results from single slices as obtained after single stimuli (SS) or after burst stimuli (TBS) on pyramidal cell activity in terms of changes of population spike amplitudes.

MLE			Pure mangiferin		
Mean ± SEM [μV]	SS 60-80 min	TBS 100-120min	Mean ± SEM [μV]	SS 60-80 min	TBS 100-120min
Control (n = 12)	-1208.72 ± 82.06	-2541.89 ± 131.94	**Control (n = 12)**	-208.72 ± 82.06	-2541.89 ± 131.94
0,10 mg/L (n = 2)	-1196.33 ± 112.00	-2619.67 ± 12.36	**0.05 mg/L (n = 2)**	-1321.33 ± 2.01	-2509.83 ± 137.24
0,30 mg/L (n = 2)	-1440.33 ± 14.38	-3201.17 ± 139.25	**0.10 mg/L (n = 2)**	-567.00 ± 76.56	-3307.67 ± 162.82
0,50 mg/L (n = 2)	-1562.33 ± 30.76	-3744.50 ± 9.19	**0.40 mg/L (n = 2)**	-129.70 ± 120.02	-3882.00 ± 29.42
0,75 mg/L (n = 2)	-2369.50 ± 19.22	-4582.33 ± 39.62	**0.70 mg/L (n = 4)**	**-653.75 ± 200.40***	**-4788.00 ± 82.75***
1,00 mg/L (n = 2)	**-1876.75 ± 38.21***	**-4167.08 ± 83.90***	**1.00 mg/L (n = 2)**	-2773.33 ± 362.41	-4557.00 ± 343.36

Overview on final results after averaging 12 slices from control and 2 slices from MLE and pure mangiferin. Data are given in microvolt ± SEM. *P*-values according to Wilcoxon, Mann und Whitney *U*-Test. *$p < 0.01$. SEM = Standard Error of the Mean

(saline 0.9% of NaCl (data not shown), as expected, while 50 mg/kg MLE, administered by gavage, resulted in a statistically significant attenuation of delta and theta spectral power in the frontal cortex and hippocampus during the first hour after administration (Fig. 8a). An increase of gamma activity emerged in the striatum and to a lesser extent in the reticular formation. These increases lasted into the fourth hour after administration. During the second hour the strongest effects were seen in the frontal cortex, consisting of a statistically highly significant attenuation of alpha1, alpha-2, beta-1a (beta a) and beta-1b (beta b) spectral power. Spectral gamma power increase reached statistical significance during the third and fourth hour in the striatum. There was no increase of motion in the video-monitored rats.

Oral administration of pure mangiferin (25.0 mg/kg) resulted in a statistically significant attenuation of theta, alpha-1, alpha-2 and beta-1a in the frontal cortex. In other brain regions, predominant attenuation of alpha-2 and beta-1a were observed. Delta power was attenuated significantly in the hippocampus and reticular formation, and consistent decrease of alpha-1 power was observed, except in the reticular formation (Fig. 8b). The duration of the increased gamma wave in the striatum was longer for MLE (4 h) than for pure mangiferin (1 h) and the increased gamma wave reached statistical significance compared to saline control after MLE application (Fig. 8a), from the 2nd to the 4th hour after administration ($p < 0.05$).

Comparison of the changes of spectral power revealed that the pattern induced by both 25 mg/kg and 50 mg/kg of MLE, administered by gavage, matched those induced by mangiferin (25 mg/kg), and this pattern of changes is comparable to that caused by caffeine published earlier (Dimpfel et al., 2018).

3.3. Psychophysiological effects in pilot translational clinical studies

3.3.1. Clinical study 1

3.3.1.1. Quantitative electroencephalogram recording. Analysis of spectral power changes in human subjects with respect to the average of all electrodes (17 surface electrodes) in the presence of MLE revealed minor changes during the recording condition “eyes open”, with attenuation of delta and theta power. However, during performance of the number sequence test (NST) significant increases of theta and beta-1 spectral power was seen ($p < 0.02$). During performance of the number connection test (NCT) beta1 and beta2 power increased (Fig. 9). In the frontal cortex there were no significant changes of spectral power across all frequency ranges, however, in the association cortex there were statistically significant changes in the spectral power of frequency ranges in response to psychometric tests (Fig. 9). The strongest effects in the association cortex emerged during performance of the number sequence (NST) with increases in frequency spectral power compared to placebo in delta ($p < 0.08$), theta ($p < 0.02$), alpha-1 ($p < 0.02$), alpha-2 ($p < 0.08$) and beta-1 ($p < 0.02$), and during the number connection test (NCT) with increases in frequency spectral power compared to placebo in delta ($p < 0.02$), theta ($p < 0.08$), alpha-1 ($p < 0.02$), beta-1 ($p < 0.08$), and beta-2 ($p < 0.08$).

3.3.2. Psychometric tests

Performance in psychometric tests in the presence of MLE did not reach statistical significance compared to placebo at 90 min after intake compared to the baseline before intake.

3.3.3. Profile of Mood States

Transient, distinct mood states were assessed with the POMS questionnaire, a standard validated psychological test formula. All 4 POMS scores (Table 2) showed an improvement 90 min after MLE intake in comparison to the baseline values, but only the score “fatigue” (S3) reached statistical significance ($p = 0.015$). The scores for “thirst for action”, “dejection” and “sullenness” all improved after intake of MLE. The placebo group did not show a trend to significant improvement in the four POMS scores.

3.3.4. Physiological parameters

There were no significant changes in heart rate variability (HRV)

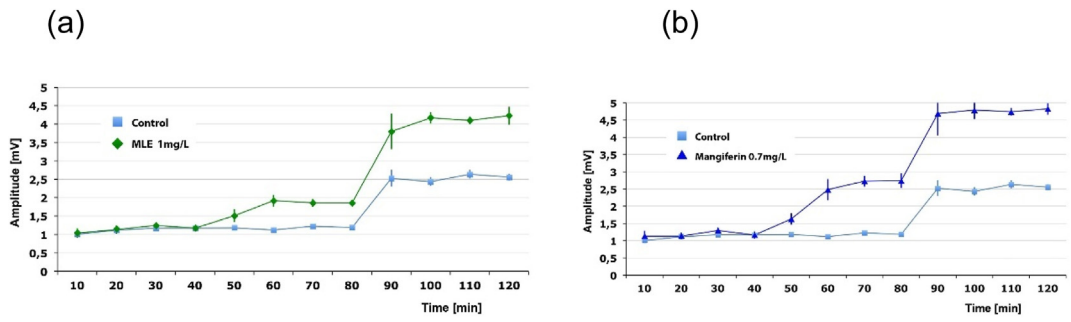


Fig. 4. Time dependent effects of control (data are given as mean ± S.E.M. of n = 12 slices) vs (a) MLE (n = 4 slices) in the presence of 1 mg/L of MLE and (b) pure mangiferin (n = 4 slices) in presence of 0.7 mg/L of pure mangiferin on pyramidal cell activity in terms of changes of population spike amplitudes (as voltage on the ordinate). Results as obtained after performance of single stimuli (10-80 min) or after burst stimuli (90-120 min). Data are given as mean ± S.E.M.

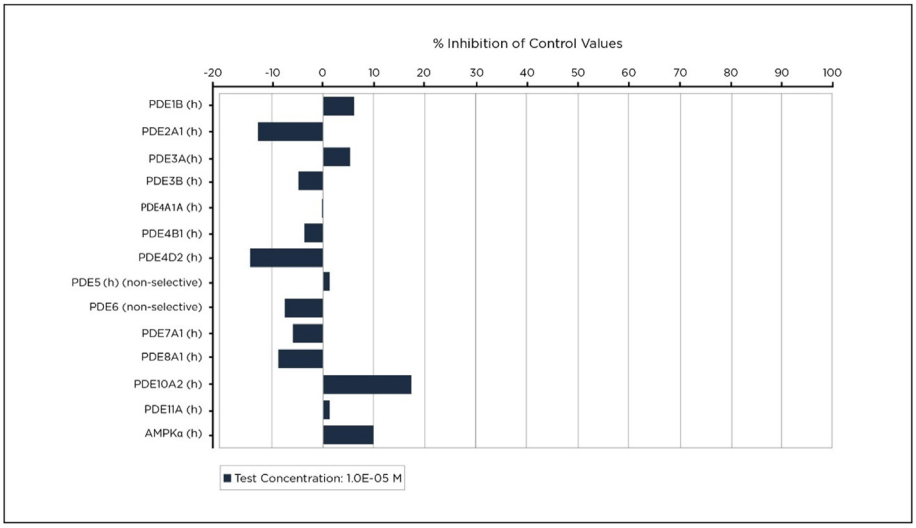


Fig. 5. Histogram of percentage inhibition of 14 enzymes of the phosphodiesterase (PDE) family.

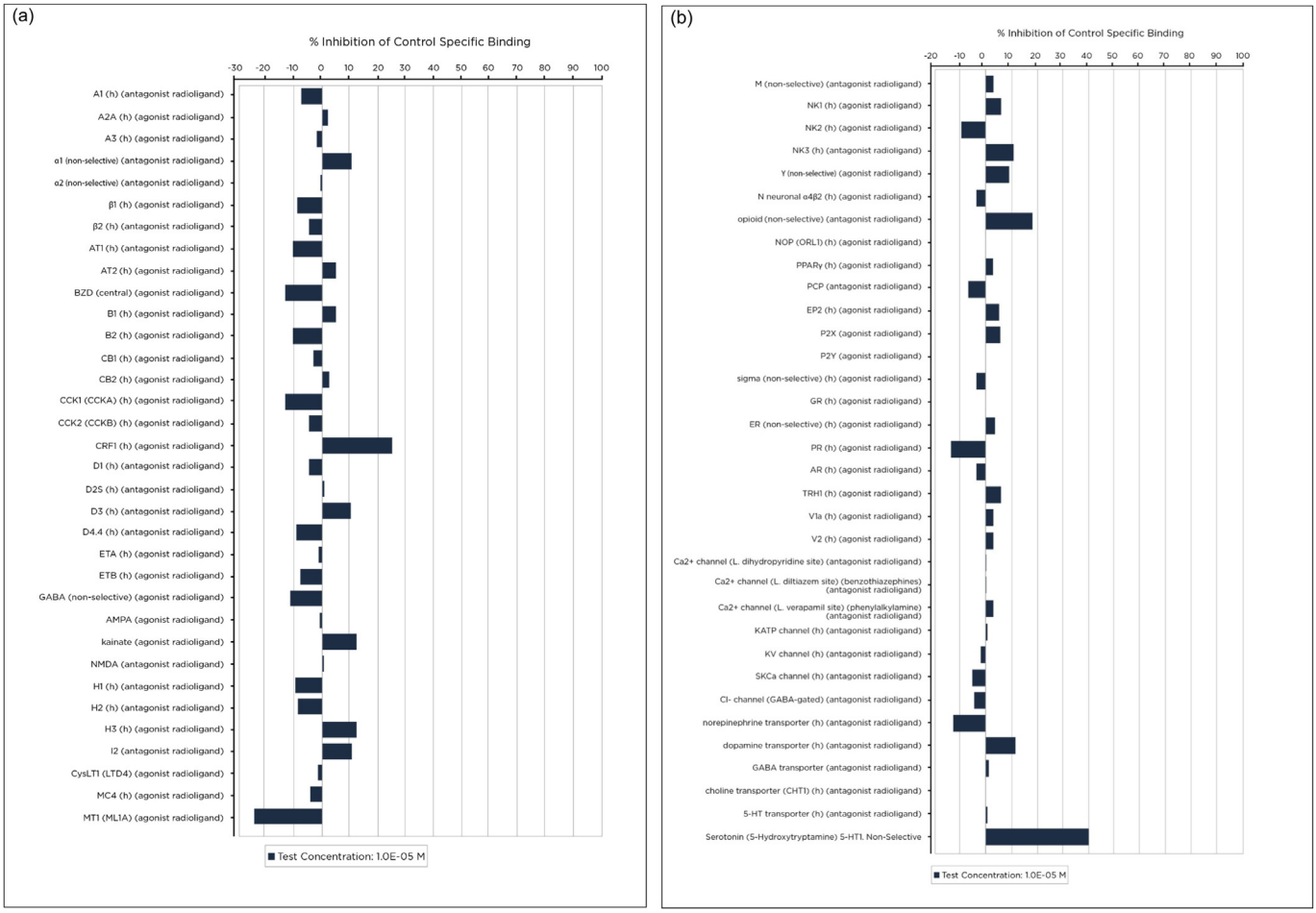


Fig. 6. (a) and (b) Histograms of percentage inhibition of control specific binding on 68 CNS targets.

(Table 3) or blood pressure (Table 4) at 90 min after intake of MLE in comparison to the baseline before intake, demonstrating that MLE does not increase blood pressure nor heart rate. MLE was given the score "very good" for tolerance by all participants.

3.3.5. Clinical study 2

3.3.5.1. Quantitative electroencephalogram recording. In order to get an overview of the results during the performed psychometric test, spectral power from all electrode positions is averaged for all recording conditions (17 scalp surface electrodes). As in the previous study, changes in power of brain waves in human subjects was observed during the performance of various tasks 60 min after intake of MLE, caffeine, or the combination. Although no significant differences were observed in the MLE group, a major change of spectral power during

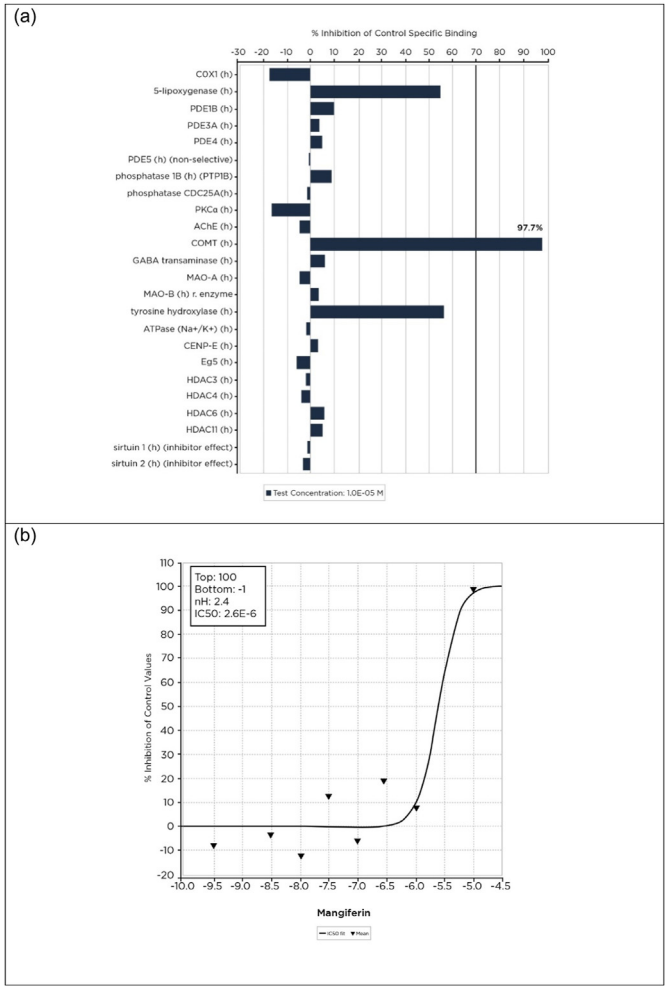


Fig. 7. Histogram of percentage inhibition of control-specific binding of 1.0E-0.5 M of pure mangiferin on 24 CNS targets. Inhibition greater than 70% was defined to represent a "hit" worth following up on with different doses (a). Log-dose-response curve of different log concentrations of mangiferin (X-axis) versus percentage inhibition (Y-axis) of catechol-O-methyltransferase (b); (IC50: 1.1 μg/ml**).**

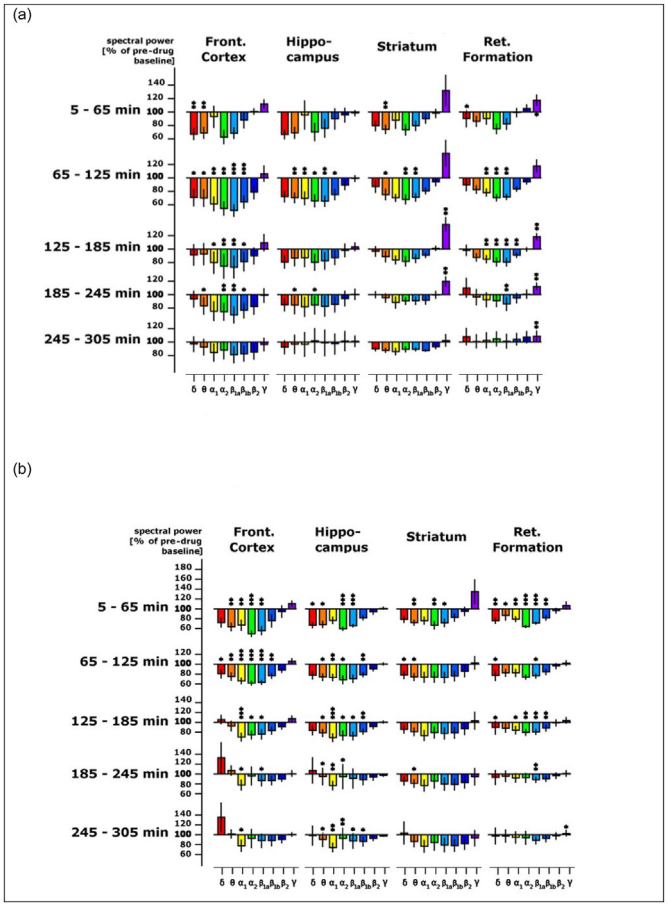


Fig. 8. Frequency changes in the presence of (a) MLE (50 mg/kg) and (b) Mangiferin (25 mg/kg). Frequency ranges are depicted as coloured bar graphs on the abscissa representing delta (red), theta (orange), alpha1 (yellow), alpha2 (green), beta a (light blue) and beta b (dark blue) and gamma spectral power (violet) from left to right within the four brain areas as mentioned on top of the graph. Statistical significance in comparison to control (vehicle) is documented by stars: * = $p < 0.10$; ** = $p < 0.05$; *** = $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

some recording conditions and in some brain regions were detected, consisting in increases (higher than 100% of baseline) of alpha1 power (yellow bar), especially during performance of the number connection test (NCT) (Fig. 10). In the presence of caffeine, significant attenuation in alpha 2 wave power (under the 100% of baseline) was observed after intake during performance of calculation (CPT) and memory test (ME). These changes occurred mainly in the frontal cortex (Fig. 10), but were not associated with a significantly improved result of the psychometric task. The combination of MLE plus caffeine showed a slight increase of alpha1 power during performance of the number connection test but no significant improvement of the task results.

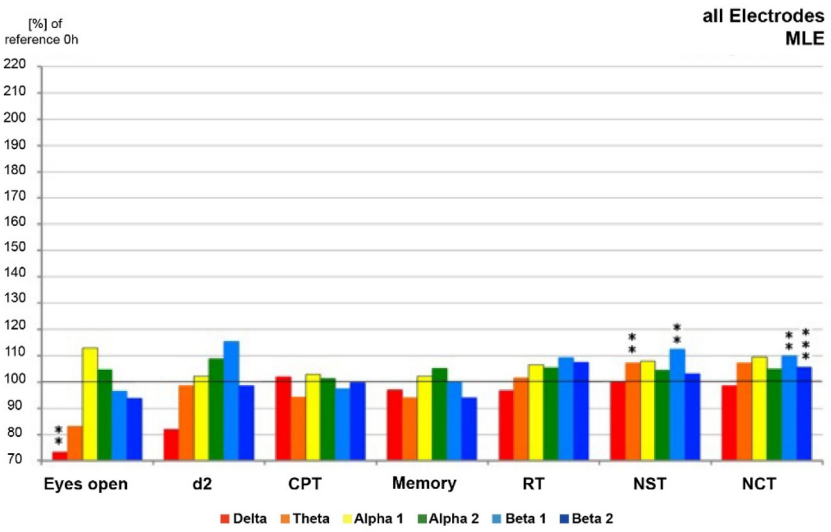
The results from acoustic (AEP) and visual P300 (VEP) obtained during psychometric testing did not change significantly 60 min after intake of MLE, caffeine, or the combination (data not shown).

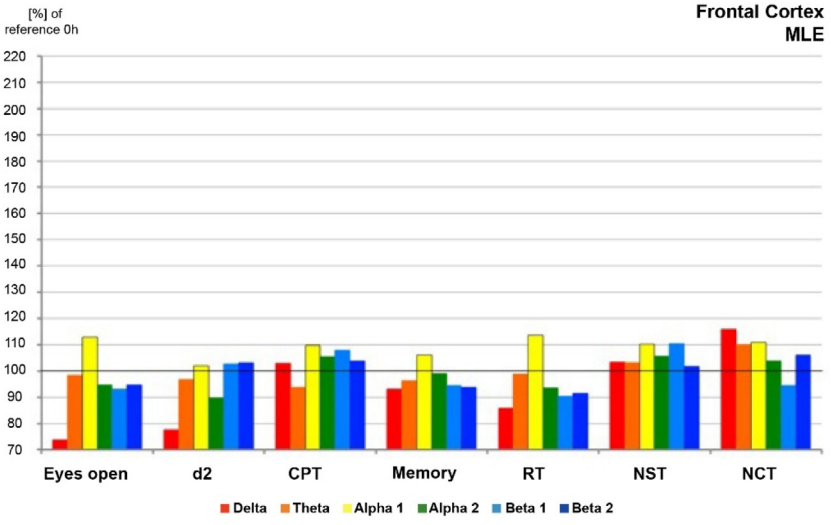
3.3.5.2. Psychometric tests. In the performance of the psychometric tests d2, CPT, ME and NCT compared to placebo, neither MLE nor caffeine reached statistically significant differences 60 min after intake compared to the baseline before intake. Within-group reaction time (RT) showed a trend towards a faster reaction time in the MLE group 60 min after ingestion ($p = 0.066$), while no such effect was found in the placebo group ($p = 0.187$) (Table 5 and Fig. 11a). Surprisingly, within group RT was significantly faster 60 min after caffeine intake or after the intake of the combination of caffeine plus MLE.

Although the RT between groups and within groups did not reach statistical significance in this small group of n = 16, Fig. 11b shows that while the placebo group reacted 5.20% slower than its baseline 60 min after intake, all three investigational products (MLE, caffeine and the combination) gave a faster reaction time compared to their baseline results, MLE 4.66% faster, caffeine 2.24% faster and the combination 4.13% faster. The percentage improvement in RT for MLE compared to placebo at 60 min was significant ($p = 0.049$). The percentage change of RT of the combination MLE plus caffeine compared to placebo was also faster at 60 min, although not significant ($p = 0.070$). There was no significant difference for the reaction time for caffeine compared to placebo at 60 min ($p = 0.438$).

3.3.5.3. Profile of Mood States. Scores of POMS in the presence of placebo or MLE did not reveal significant differences at 60 min in comparison to baseline before intake, while caffeine intake decreased the score for "dejection" (0h: 0.26 (0.50) vs 60 min (0.22 (0.41), $p = 0.05$).

3.3.5.4. Physiological parameters. MLE was given the score "very good" for tolerance by all participants. As with study 1, there were no significant changes in blood pressure or heart rate (Table 6) at





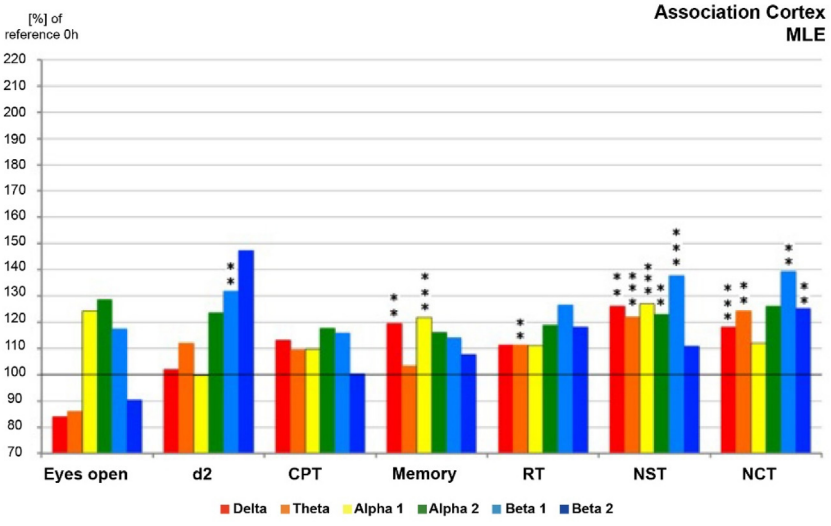


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Fig. 9. Spectral frequency changes in % of the baseline after intake of MLE in the relaxed state (eyes open) and during performance of six cognitive demands. Data are documented as median of all electrode positions (upper graph), frontal cortex (middle graph) and associative cortex (lower graph). Red colour: delta; orange: theta; yellow: alpha1; green: alpha2; turquoise: beta1 and blue: beta2 spectral power concentration. Cognitive tests: Concentration test (d2), memory test (ME), calculation performance test (CPT), reaction time test (RT), number sequence test (NST) and number connection test (NCT). Statistical significance (Sign-Test) between placebo and MLE is indicated by stars. * = $p < 0.11$; ** = $p < 0.08$ and *** = $p < 0.02$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Results from the questionnaire Profile of Mood States (POMS) at visit day before and after intake of Placebo or MLE.

TEST	Placebo				MLE			
	Mean (SD)				Mean (SD)			
	S1	S2	S3	S4	S1	S2	S3	S4
0 min (Baseline)	0.60 (1.05)	0.50 (0.91)	2.05 (1.24)	2.54 (1.46)	0.48 (0.74)	0.27 (0.46)	2.10 (1.10)	2.05 (0.96)
90 min after intke	0.29 (0.57)	0.20 (0.43)	0.37 (0.99)	2.72 (1.39)	0.11 (0.34)	0.14 (0.34)	1.12 (1.04)	2.72 (1.11)
p (0 min vs 90 min)	ns	ns	ns	ns	ns	ns	0.015	ns

Four mood states were used for the evaluation of the POMS questionnaire: S1 = "dejection", S2 = "sullenness", S3 = "fatigue", S4 = "thirst for action", SD = standard deviation. ns = not significant. Statistical significance was calculated by Wilcoxon test.

Table 3
Heart rate variability (HRV) from electrocardiographic recordings in the relaxed state. Frequency is given in Hz.

Time (minutes)	0 (min)	90 (min)
Heart Rate (SD)	70.20 (12.43)	65.76 (10.20)
Standard deviation in N-N intervals (SD)	63.69 (36.03)	64.51 (32.92)
Root Mean Square of successive differences (SD)	63.44 (48.97)	70.41 (45.98)

Results of heart rate is given in bpm (beats per minute). SD = standard deviation.

Table 4
Overview measurements of blood pressure (mmHg) and heart rate (bpm) for MLE.

	SBP(SD)	DBP (SD)	Pulse (SD)
1st record	118.06 (14.8)	75.88 (10.47)	68.75 (13.86)
2nd record	118.44 (14.61)	74.38 (11.32)	71.94 (12.39)
3rd record	119.56 (17.40)	75.19 (11.95)	67.81 (11.88)
4th record	118.38 (16.77)	75.00 (12.89)	64.88 (13.50)

SBP = systolic blood pressure; DBP = diastolic blood pressure; SD = standard deviation. 1st record = baseline, before the first qEEG-recording, 2nd record = 20 min after the first EEG-recording, 3rd record = before the second EEG-recording (90min after intake of MLE) and 4th = 20 min after the second EEG-recording (90 min after intake of MLE).

90 min after intake of MLE in comparison to the baseline before intake.

4. Discussion

4.1. Hippocampal slice model

Measuring the increase or decrease of amplitude of population spikes in response to a single stimulus and theta burst electrical stimuli in prepared hippocampal slices provides a model of hippocampal pyramidal cell response to investigative compounds (Dimpfel et al., 2016). Results may be extrapolated to effects on long-term synaptic plasticity in relation to memory (Kullmann and Lamsa, 2007). Both isolated mangiferin and MLE increased the excitability of hippocampal pyramidal cells in a similar pattern under both single-stimulus (from 10 to 80 min) and theta burst stimuli (from 90 to 120 min).

It can be concluded that mangiferin, the major compound in MLE, is the main active compound responsible for increasing pyramidal cell excitability in the hippocampus and has the potential to enhance spatial and time-dependent memory (Dimpfel, 2015). Pyramidal cell excitability is mediated by glutamatergic signaling from the stimulated Schaffer collaterals, and it can be concluded that both, mangiferin and MLE, modulate this glutamatergic signaling in the hippocampus. A recent publication (Dimpfel et al., 2018) demonstrated, in an in-vitro hippocampal slice model, that MLE given by gavage changes the excitability of the hippocampus of rats in a similar way to caffeine, and the present study demonstrates that it is mangiferin that is mainly responsible for this activity.

4.2. In-vitro studies of mangiferin on CNS targets

In-vitro screening against 106 CNS receptor, enzyme and transporter targets demonstrated, that isolated mangiferin inhibits COMT, and in the functional assay this activity was found to have a moderately potent IC_{50} of 1.1 µg/ml. Apart from COMT inhibition, the assays revealed no significant (a significant "hit" being defined as greater than 70% inhibition of control specific binding) inhibitory activity on any other receptor, enzyme, or transporter, including serotonin, dopamine, phosphodiesterases and MOA-A and MOA-B, even though mangiferin has previously been reported to inhibit MAO (Bhattacharya et al., 1972). Mangiferin was not found to act as an adenosine receptor antagonist or PDE4 inhibitor, which are the main mechanisms of action of caffeine. Thus, although MLE and mangiferin have similar effects on brain electrical activity to caffeine in rats, this effect is mediated by a different mechanism of action to caffeine. Thus, one can expect mangiferin and MLE to have a different side-effect profile to caffeine.

This is the first time to our knowledge, that mangiferin has been demonstrated to be a COMT inhibitor. COMT is the magnesium-dependent intracellular enzyme that catalyzes the transfer of a methyl group from the common methyl donor *S*-adenosyl-*L*-methionine to substrates incorporating a catechol structure (Kiss and Soares-Da-Silva, 2014). COMT's main physiological function is the metabolic inactivation of endogenous catechol neurotransmitters, including dopamine, epinephrine, and norepinephrine. Inhibition of COMT activity modulates the delicate balance of dopamine and norepinephrine signalling, and influences cortical information processing (Dickinson and Elvevåg, 2009). Dopamine is a neurotransmitter involved in regulation of mood, craving and reward, and in the control of movement and coordination. Pathological reduction of dopamine levels in the midbrain is associated with degenerative neurological disorders such as Parkinson's Disease and COMT inhibitors are currently used to extend the duration of action of the pharmaceutical L-dopa in the management of Parkinson's Disease (Kiss and Soares-Da-Silva, 2014).

Since COMT is the primary dopamine metabolizing enzyme in the prefrontal cortex, a region where dopamine mediates cognitive functions including working memory, planning and attention (Dickinson and Elvevåg, 2009), COMT inhibition enhances dopamine signalling to stabilize and protect information acquisition and processing (Dickinson and Elvevåg, 2009; Seamans and Yang, 2004). Considerable evidence has implicated involvement of the dopaminergic system in the prefrontal cortex in the pathology of Attention Deficit Hyperactivity Disorder (ADHD), and the potential role of COMT inhibitors as potential therapeutics in ADHD (Sun et al., 2014). In healthy volunteers, the

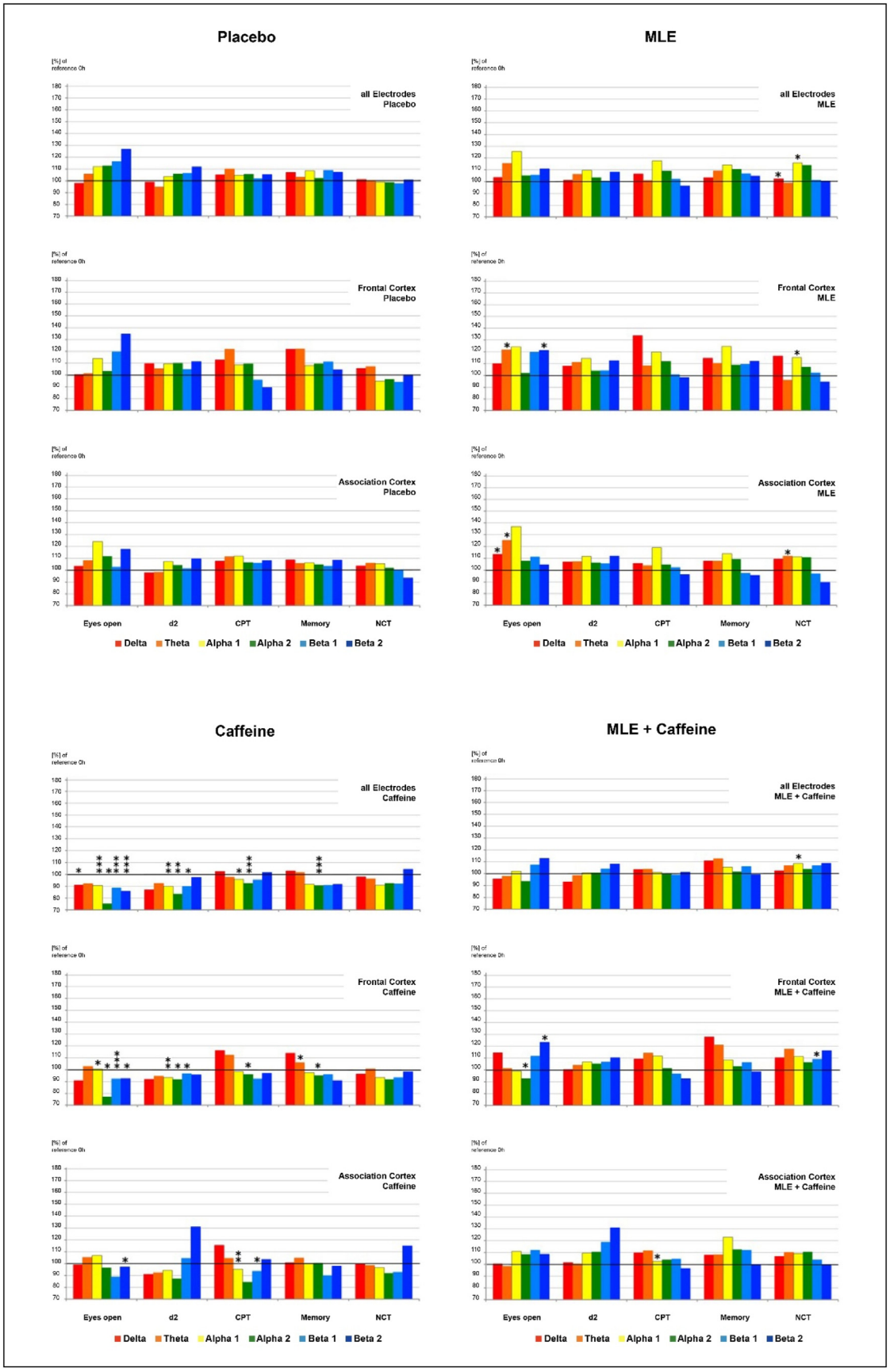

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Fig. 10. Spectral frequency changes in % of the baseline after intake of placebo (a), MLE (b), caffeine (c), or the combination of MLE + caffeine (d) in the relaxed state (eyes open) and during performance of four cognitive demands. Data are documented as median of all electrode positions (upper graph), frontal cortex (middle graph) and association cortex (lower graph). Red colour: delta; orange: theta; yellow: alpha1; green: alpha2; turquoise: beta1 and blue: beta2 spectral power concentration. Cognitive tests: concentration test (d2), calculation performance test (CPT), memory test (ME) and number connection test (NCT). Statistical significance (Sign-Test) between placebo and products are indicated by stars. * = $p < 0.10$; ** = $p < 0.05$ and *** = $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

COMT inhibitor tolcapone has shown benefit in improving executive functioning and the efficiency of cortical information processing (Apud et al., 2007). Both mangiferin and MLE warrant future clinical studies on attention, learning, and memory in healthy individuals and as well as in a clinical population with ADD and ADHD.

In addition to potential applications in enhancing cognitive functions, COMT inhibitors, by enhancing the availability of dopamine in the prefrontal cortex and related reward circuitry of the brain, can potentially reduce craving for several substances of abuse, and reduce negative reward-seeking behaviours. For example the COMT inhibitor tolcapone has been shown to reduce ethanol intake in alcohol preferring rats (McCane et al., 2014), the COMT inhibitor entacapone reduced craving for cocaine in two case studies (Rodrigues-Silva and Vasconcelos, 2016), and in an eight week trial in twenty four subjects with pathological gambling, tolcapone reduced symptoms of pathological gambling (Grant et al., 2013). The potential for MLE and mangiferin to reduce craving or attenuate substance seeking behaviour should be explored in future clinical studies, as this may have public health implications, for example applied to nicotine cessation programs, weight management programs and substance abuse rehabilitation.

While the bioavailability of mangiferin has been shown in a human pharmacokinetic study to be only 1% of the intake dose (Hou et al., 2012), the activity of mangiferin and MLE on COMT may be extended by the presence in MLE of isomangiferin and homomangiferin, and by the mangiferin aglycone metabolite norathyriol, which have the same catechol structure as mangiferin.

4.3. Changes in brain electrical activity in-vivo by qEEG

From the in-vivo qEEG studies we can conclude that both mangiferin and MLE change brain electrical activity in a similar way, attenuating EEG spectral power in the frontal cortex, hippocampus and to a lesser extent the hippocampus and striatum. This pattern of attenuated spectral power activity across multiple frequency ranges recorded from implanted brain electrodes, is a brain stimulatory signature, which is very similar to that shown for caffeine (Dimpfel et al., 2018). Mangiferin is the major CNS bioactive compound in MLE, and is absorbed and bioavailable. Mangiferin, and/or a mangiferin metabolite such as norathyriol, also crosses the blood brain barrier in sufficient concentrations to alter brain electrical activity by changes in regional neurotransmitter signalling.

Regarding mechanisms of action, in the in-vitro hippocampal slice model both mangiferin and MLE increased the amplitude of the pyramidal cell population spike, which is mediated by glutamate signalling from the Schaffer collaterals. With the broad in-vitro binding studies of CNS targets, it was discovered that mangiferin is a COMT inhibitor, and thus enhanced dopaminergic and nor-adrenergic signalling can be expected. According to the in-vivo qEEG results for mangiferin in the frontal cortex during the first hour, statistically significant attenuation of the frequency ranges theta, alpha-1, alpha-2 and beta1a

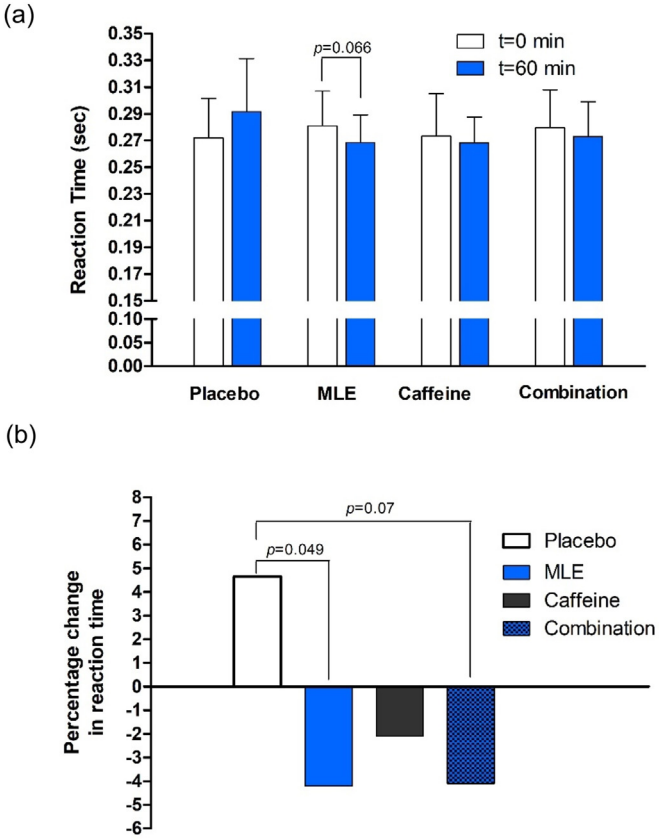

Fig. 11. Comparison of reaction time (RT) in seconds for placebo, mango leaf extract (MLE) caffeine and MLE-caffeine combination (a). Percentage of change in RT for placebo, MLE, caffeine and MLE plus caffeine combination (b). Significance between groups was calculated by Wilcoxon signed-rank test.

Table 5
Psychometric performance during several tests and measurement of reaction time (RT given in sec) for placebo, mango leaf extract (MLE), caffeine and MLE + caffeine.

Test	placebo		MLE		caffeine		MLE + caffeine	
	0 min	**90 min**	**0 min**	**90 min**	**0 min**	**90 min**	**0 min**	**90 min**
d2-Test (SD)	14.30 (2.26)	15.08 (2.39)	15.32 (3.23)	16.20 (3.18)	13.96 (3.47)	15.89 (3.10)	14.75 (2.92)	15.75 (2.89)
CPT (SD)	2.58 (1.85)	3.73 (3.80)	3.89 (4.44)	3.40 (4.00)	3.76 (2.89)	3.75 (3.10)	3.20 (3.92)	3.37 (3.75)
Me-test (SD)	7.54 (1.95)	8.27 (2.28)	8.86 (2.10)	8.42 (2.13)	8.90 (1.94)	8.14 (2.39)	7.59 (2.04)	8.37 (2.56)
NCT (SD)	192.96 (34.86)	198.08 (39.57)	199.34 (44.43)	203.32 (60.77)	189.07 (40.06)	199.96 (40.39)	189.25 (36.21)	189.15 (37.15)
RT-test (SD)	0.272 (0.051)	0.291 (0.069)	**0.281* (0.045)**	**0.269* (0.036)**	0.273 (0.055)	0.268 (0.033)	0.280 (0.049)	0.274 (0.064)

Psychometric tests: concentration test (d2), calculation performance test (CPT), memory test (ME) and number connection test (NCT), and reaction time (RT) in milliseconds. Results are presented as mean of the result (SD). SD = standard deviation. Statistical significance between placebo and treatment groups were performed by Wilcoxon test.

Table 6
Blood pressure (mmHg) and heart rate (bpm) for MLE.

	SBP(SD)	DBP (SD)	Pulse (SD)
1st **record**	120.50 (13.77)	78.31 (9.57)	71.50 (10.89)
2nd **record**	119.63 (14.79)	73.00 (6.31)	72.13 (9.86)
3rd **record**	121.69 (15.81)	77.00 (12.12)	66.31 (10.40)
4th **record**	114.69 (13.96)	72.69 (7.74)	66.94 (10.56)

SBP = systolic blood pressure; DBP = diastolic blood pressure; SD = standard deviation. 1st record = baseline, before the first EEG-recording, 2nd record = 20 min after the first EEG-recording, 3rd record = before the second EEG-recording (1h after intake of MLE) and 4th = 20 min after the second EEG-recording (1h after intake of MLE). Pulse: heart rate (beats per minutes (bpm)).

can be seen. It has been suggested that that the major neurotransmitter systems underlying these frequency ranges are respectively nor-adrenalin (theta waves), serotonin (alpha-1 waves), dopamine (alpha-2 waves) and glutamate (beta-1 waves) (Dimpfel, 2015). The modulation of theta waves and alpha-2 waves may be partly due to the effect of mangiferin as a COMT inhibitor enhancing noradrenergic and dopaminergic signalling.

Gamma waves were induced by MLE mainly in the striatum, which lasted for 4 h, and reached statistical significance ($p < 0.05$) from the 2nd to the 4th hour after administration. There are several papers dealing with the gamma activity of local field potentials in the literature, with contributions that relate gamma activity to movement (Masimore et al., 2005), and others that relate gamma activity to attention, learning, and memory (Dimpfel and Biller, 2015; Dimpfel and Schombert, 2015). Since there was no concomitant increase of motion of the animals, confirmed through video monitoring, the increased gamma activity is not due to movement of the animals but may be an indicator of a state of increased alertness and attention in the animals.

4.4. Psychophysiological effects in pilot translational clinical studies

4.4.1. qEEG recording

In each of the two pilot single dose randomised double-blind, placebo controlled clinical pilot trials, the effect of MLE on qEEG was studied at two different time-points (90 min after intake in study 1, and 60 min after intake in study 2). In study 1, compared to placebo, there were significant increases in changes in qEEG spectral power across several frequency ranges 90 min after only a single dose of MLE, with the strongest effects in the association cortex during cognitive challenges presented by the NST and the NCT. As in study 1, some changes in the power of brain electrical activity were observed during the performance of the different psychometric test 60 min after intake of MLE, caffeine and the combination. Although no significant differences were observed in the MLE group, a major change of spectral power during some recording conditions and in some brain regions were detected, consisting in increases of alpha-1 power (yellow bar), especially during performance of the NCT. In the presence of caffeine, significant attenuation in alpha 2 wave power was observed after intake during performance of calculation (CPT) and memory test. These changes occurred mainly in the frontal cortex.

Taken together, the results of the in-vivo and two pilot clinical studies show that compared to placebo, a single dose of MLE changes brain electrical activity, and that active compounds, primarily mangiferin, and/or its metabolite/s, are absorbed, are bioavailable, and can cross the blood brain barrier in sufficient concentration to effect changes in the underlying neurotransmitters responsible for this brain electrical activity. To our knowledge these are the first qEEG studies conducted on mangiferin in-vivo and on MLE both in-vivo and in human RCT studies.

4.4.2. Psychometric tests

In study 1, performance in psychometric tests in the presence of placebo or MLE did not reach statistically significant differences at 90 min after intake compared to the baseline before intake. In study 2, neither MLE nor caffeine reached statistically significant differences in the performance of the psychometric tests d2, CPT, ME, and NCT, compared to placebo, 60 min after intake. Within group RT was faster in the MLE group 60 min after ingestion ($p = 0.066$), while this effect was not detected in the placebo group ($p = 0.187$). Within group RT was not significantly faster 60 min after caffeine intake or after the intake of the combination of caffeine plus MLE. The percentage improvement in RT for MLE compared to placebo at 60 min was significant ($p = 0.049$).

These exploratory psychometric tests are disappointing, however the faster RT test for MLE, and the lack of significant activity on RT for caffeine, indicate that the effect of both these test substances on psychometric tests need to be explored further in better powered larger clinical studies. Caffeine has been reported to give a faster reaction time in non-fatigued subjects (Santos et al., 2014). MLE may need to be given in repeat doses for blood levels of mangiferin and/or active metabolites to reach a steady state and have an impact on the other psychometric tests in the test battery.

4.4.3. Profile of Mood States questionnaire

In study 1, 90 min after intake of MLE, all 4 of the POMS scores showed an improvement in comparison to their baseline values, with the score for “fatigue” reaching statistical significance ($p = 0.015$). The placebo group did not show a trend to significant differences in the four POMS scores. The effect of MLE on fatigue and mood should be explored further in a larger study of longer duration, preferably in mentally or physically fatigued subjects, and subjects with mild to moderate depression. The significant change in POMS score for fatigue accords with the folk uses of infusions of *Mangifera indica* leaf for fatigue and as a tonic, referenced in the introduction to this paper. In study 2, none of the four the POMS scores for MLE showed significant differences at 60 min in comparison to the baseline scores.

4.4.4. Physiological parameters

In both studies MLE was very well tolerated, and there were no changes in blood pressure, heart rate variability 90 min after intake of a single 500 mg dose of MLE (study 1) or blood pressure or heart rate 60 min after intake of the 500 mg single dose of MLE (study 2).

The two double-blind, randomised placebo controlled translational studies are limited by being single-dose exploratory studies in a small number of healthy subjects, and future studies investigating the CNS effects of MLE should be done in larger populations.

5. Conclusions

The present series of studies have demonstrated for the first time that mangiferin is the major CNS-active compound in MLE, is a COMT inhibitor, and has no significant activity on the adenosine receptors and phosphodiesterase 4 enzymes, main targets for the CNS activities of caffeine.

While limited by being single-dose studies in a small number of healthy subjects, the two translational clinical studies are the first human studies to provide evidence that MLE is well tolerated, has no effect on blood pressure, pulse or heart rate variability, and compared to placebo can alter brain electrical activity during cognitive challenges, give a faster reaction time, and give a decreased score for fatigue in the POMS questionnaire.

The CNS activities of the present studies provide a rationale for the use of mango leaf tea as a substitute for tea reported a century ago (MacMicking, 2007), and for the traditional uses of infusions and decoctions of mango leaf for fatigue and exhaustion (Doughari and Manzara, 2008).

Larger controlled clinical studies are needed for extract *Mangifera indica* L., Zynamite, to realise the potential of this ingredient to be

recognized as a new generation of natural caffeine-free nootropic for the food, beverage and supplement industries.

Availability of data materials

Data are all contained within the paper.

Author contributions

NG and JCW conceptualized, planned and directed the research, LLR project managed the research. TVM prepared the MLE and the standardization by UPLC to the content of mangiferin. All four authors contributed to writing, preparing figures and reviewing the manuscript.

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Declaration of competing interest

Laura López-Ríos, Tanausú Vega-Morales and Julia C Wiebe are employed by the sponsor of the reported series of studies, Nektium Pharma SL. Nigel Gericke works as a consultant to Nektium Pharma. Nektium Pharma produced the *Mangifera indica* extract, with production, standardization and analytical work on the extract done by Vega-Morales, while López-Ríos, Wiebe and Gericke designed and interpreted the studies. None of the authors had a role in the actual experimentation or data collection.

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Abbreviations

MLE	*Mangifera indica* leaf extract
LTP	long-term potentiation
EEG's	electroencephalographies
qEEG	quantitative electroencephalography
COMT	catechol-O-methyltransferase
POMS	Profile of Mood States
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
MAO	monoamine oxidase
BNDF	brain-derived neurotrophic factor
LPS	lipopolysaccharide
NLRP3	NLR Family Pyrin Domain Containing 3
ASC	inflammasome adaptor protein
NOAEL	no observed adverse effect level
GRAS	generally recognized as safe
ACSF	artificial cerebrospinal fluid
SS	single stimuli
TBS	theta burst stimuli
ARS	adenosine receptors
PDE	phosphodiesterase
FFT	fast fourier transformation
D2	d2 attention test
CPT	Calculation performance test
ME	memory test
RT	Test reaction time test
NST	number sequence test
NCT	number connection test

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.112996.

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